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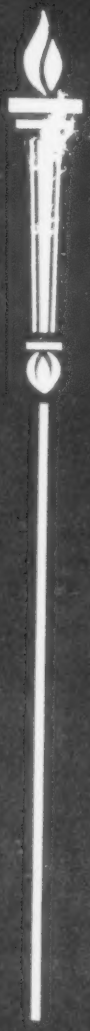

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George Hall Hogeboom

1913–1956

Late Head of the Cellular Biology Section

Laboratory of Biology, National Cancer Institute

George Hall Hogeboom

1913–1956

The sudden death of George Hall Hogeboom on July 5, 1956, following a minor operation, came as a great shock to his family, his colleagues, and his friends. Although he had passed his forty-third birthday only a few months earlier, George had already achieved international recognition for his fundamental work in cytochemistry. Indeed, his contributions to the elucidation of cell function by the isolation and biochemical characterization of subcellular structures were so important that his name will undoubtedly go down in modern scientific history as one of the founders of this comparatively new and highly active branch of cytochemistry. His death, at the zenith of his career, is a great loss.

George was born on April 26, 1913, in Springfield, Missouri, and spent a memorable boyhood there. He loved Springfield and the nearby Ozarks deeply, and despite the fact that he came to know many other places, both in this country and abroad, he always harbored a desire to settle down in Springfield some day. He had early contact with the practice of medicine through his father, who was a well-known surgeon, but because his father died of cancer he decided to devote his life to cancer research rather than to medical practice.

George received his early education at Washington University in St. Louis. Despite the fact that he planned to study medicine later, he was greatly interested in chemistry and made this his major field. He was graduated from Washington University in 1935 and entered The Johns Hopkins University School of Medicine, from which he received his medical degree in 1939. He became a member of Phi Beta Kappa while at Johns Hopkins and was also the youngest man ever to be admitted to Alpha Omega Alpha, the honorary medical society. After serving as

an intern at The Johns Hopkins Hospital, he was awarded the William Stewart Halsted Fellowship in Surgery and, under the aegis of Professor A. R. Rich, was placed in charge of the Surgical Pathology Laboratory.

His entrance into research came in 1941, when he was appointed to the staff of the Rockefeller Institute for Medical Research in New York and was assigned to the laboratory of Dr. James B. Murphy, one of the pioneers in cancer research. Following a biochemical study of the enzymes involved in the production of melanin in a mouse melanoma, the war intervened, and from 1942 to 1945 George was called upon to do research on mustard gas and other vesicants by the Office of Scientific Research and Development. Later he served with the Dugway Proving Ground Mobile Chemical Warfare Service Unit in Florida as chief of the laboratory section and assistant to the commanding officer. Here he was responsible for the complete reorganization of the laboratory into an efficient working unit.

Upon his return to the Rockefeller Institute, George was drawn into research problems of wide scope. He collaborated on the isolation and purification of antibiotics and on the separation and biochemical characterization of subcellular tissue components. He was also actively interested in tissue culture and in the examination of such cultures and of isolated subcellular structures with the electron microscope.

His main research interest, however, and the one that was to engross him for the rest of his life, was concerned with the isolation of subcellular structures from tissues. In this field he contributed much to both theory and practice. The work for which he is best known resulted from the discovery in 1947 that, by using hypertonic solutions of sucrose, morphologically and cytologically intact mitochondria could be isolated from liver tissue. Furthermore, he was able to show that many of the important respiratory enzymes of the liver cell were localized exclusively in the isolated mitochondria. These findings gave substance to speculations that cytologists and cell physiologists had entertained for decades; namely, that these ubiquitous intracellular granules were the center of respiration and energy production in the cell. The experiments were, in effect, a wedding of the sciences of cytology and biochemistry and laid a firm foundation for a new branch of cytochemistry called cell fractionation. Today, cell fractionation, in one form or another, is being being used by literally hundreds of scientists throughout the world to study the chemical composition and biochemical functions of mitochondria and other subcellular structures under normal and pathologic conditions.

In 1948, at the behest of Dr. Harry Eagle, then Scientific Director of the National Cancer Institute, George joined the staff of the Institute to organize a new laboratory to be devoted to cell-fractionation studies of normal and malignant tissues. He served as head of this unit until his death. While at the National Cancer Institute he continued his experiments on mitochondria, making extensive studies of their enzymatic functions, chemical composition, and physical organization. This work was also extended to liver tumors, and it was found that these tumors were greatly deficient in mitochondria and in the enzymes and other proteins that these granules normally contained. One of his most important recent accomplishments was the discovery that one of the key coenzymes involved in cellular oxidations and reductions, diphosphopyridine nucleotide, was synthesized exclusively in the nucleus of the cell. This is the first nuclear function to be defined in precise biochemical terms.

In recent years, George was unable to devote as much time to laboratory work as he would have liked. Although he was primarily a laboratory man, the constant invitations to lecture and to review progress in his field made serious inroads on the amount of time he could spend in the laboratory. Four years ago, he was incapacitated by tuberculosis, but reached complete recovery following treatment and surgery. At the time of his death, he had completed plans to lecture in Copenhagen and to spend a sabbatical at the University of Brussels with Professor Albert Claude, with whom he had collaborated at the Rockefeller Institute.

Among the qualities which contributed to George's success as a scientist and as a man was his remarkable ability to penetrate to the heart of a problem. He was quick to detect false or misleading ideas and inconclusive experimental approaches. Once he had reached a decision about an experiment, he would "take the bull by the horns," as he was fond of saying, and spare no effort in its completion. As a result, his experiments were characterized by a thoroughness that has made his findings withstand the test of time. George was also outspoken. He did not believe in mincing words and did not hesitate to criticize others. But he was at least as critical of himself as he was of other people and was always the first to point out the flaws and limitations of his own work. His honesty and sincerity earned the respect of all who knew him. George was also noted for his willingness to help other people. He was always ready to listen to someone else's troubles and to offer sound advice. He gave generously of his time in the service of others and did not hesitate to offer more tangible assistance whenever he could. Although he led a fairly large group of junior and senior investigators, he not only scrupulously avoided directing their research, but also vigorously championed their right to work independently.

George met his wife, Leslie, at the Rockefeller Institute where she was working as a secretary and medical technician in the same laboratory. They were married in 1942, and Leslie developed and maintained a deep and active interest in George's work throughout their marriage. George and Leslie had four children: Jan, Gail, George, Jr., and Thomas. Outside the laboratory George had many interests. He was a connoisseur of good food, drink, and music and loved to play poker. He and several of his colleagues met every other Friday evening to match wits in the game. George was also fond of sports of all kinds but his favorite was golf. He was an exceptionally good golfer and at one time was the Missouri Amateur Champion. Although he had few opportunities to play during the last few years, when he did, he managed to score in the 70's or low 80's with little effort.

Those of us who were fortunate enough to have the privilege of working with George have felt his loss keenly. We will especially miss the frequent and informal chats we had with him, his uninhibited expressions, his understanding, his courage, and his generosity.

Walter C. Schneider

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Histochemical Characteristics of Intrasplenic Ovarian Transplants in Gonadectomized Rats [1,2]

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The application of a group of histochemical techniques to normal rat ovaries in different phases of the estrous cycle established the value of such methods for identifying the various tissue components of the ovary and for assessing their physiologic activity (*2*). In the present study, several of these same methods have been applied to experimentally induced ovarian tumors. The tumors were produced by transplanting pieces of ovary into the spleens of gonadectomized rats. It is believed that transfer of ovary to a site drained by the portal vein results in inactivation of most of the ovarian hormones by the liver before they reach the general circulation (*3*). In consequence, there is an increased output of gonadotrophins by the anterior pituitary, and, under this influence, the transplanted ovarian tissue enlarges and eventually loses its normal organization (*4–14*).

Materials and Methods

Male and female Sprague-Dawley (Hisaw) rats, approximately 2 months old, were gonadectomized, and 1 ovary was inserted beneath the capsule on the lateral aspect of the spleen. Two groups of control animals were employed: (*a*) ovariectomized female rats in which the transplanted ovary was placed beneath the kidney capsule, and (*b*) others in which the implant was placed deep in the left lobe of the liver.

At approximately bimonthly intervals for most of the females, but at monthly intervals for those killed within a few months after operation, vaginal smears were made for 5 successive days in order to determine whether the hosts displayed a persistent anestrous smear or showed periodic vaginal cornification. When cornification was observed, it was assumed that there was a considerable amount of circulating estrogen (*15*).

The 12 rats bearing transplants to the kidney were killed after 1 to 10½ months. The 5 rats with transplants to the liver were killed after 3 to 6

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[2] Supported in part by an institutional grant to Harvard University from the American Cancer Society, Inc. A preliminary report was read before the American Association of Anatomists (*1*).

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[4] Research carried out during the tenure of a Lederle Medical Faculty Award.

[5] *Present address:* Department of Anatomy, Cornell University Medical College, New York, N. Y.

[6] We are especially indebted to Mrs. Pauline R. Noble for assistance in the care of the animals and for making most of the histochemical preparations. Mr. Leo J. Talbert prepared the photomicrographs.

months. Forty-four rats with intrasplenic grafts were killed after 1 to 14 months. When killed, each animal was weighed and its ovarian graft measured. In females, the uterus was also weighed. The approximate volume of the transplant was calculated by using the formula for an oblate spheroid: ½ × length × breadth × depth.

Histologic procedures.—The ovarian graft, if sufficiently large, was divided into 4 pieces, which were fixed, respectively, in the Zenker-acetic acid mixture, in 10 percent neutral buffered formalin, in Orth's or Rossman's fluid (picric acid-alcohol-formalin), and in chilled 80 percent ethanol. Smaller grafts were fixed only in Zenker's fluid and formalin. From females, segments of the uterus were fixed in Zenker's fluid, in Orth's or Rossman's fluid, and in chilled 80 percent ethanol.

Blocks fixed in Zenker-acetic were embedded in paraffin and sectioned at 5μ. Representative sections were stained with hematoxylin and eosin, with a dilute solution of eosin and methylene blue buffered to *p*H 5.13 (*16*), and with Masson's connective-tissue stain.

Blocks fixed in formalin for 1 week were washed overnight and sectioned at 15μ on the freezing microtome. Sections were stained for lipides with 1 percent Sudan black B in 70 percent ethanol for 7 minutes, for carbonyl groups by a modified Ashbel and Seligman hydrazide method (*17*), and for cholesterol by the Schultz method. Control sections were first extracted for half an hour with acetone at room temperature.

Blocks fixed in Orth's or Rossman's fluid were embedded in paraffin, cut at 5μ, and stained by the periodic acid-Schiff technique for carbohydrate-containing substances. Glycogen was removed from control sections by treatment with saliva or diastase before staining.

Blocks fixed in 80 percent ethanol were embedded in paraffin, sectioned at 5μ, and stained for the demonstration of alkaline-phosphatase activity (*p*H 9.5). Slides were incubated for 5 hours in media containing glycerophosphate or yeast nucleic acid. Long incubation of this sort permits tissue, but not intracellular, localization of activity.

Results

Female genital tract.—Periodic vaginal cornification and large uteri characterized all the animals with successful grafts to the kidney and liver. This had been expected for those with kidney transplants, since venous drainage from that site is outside the portal system, but was unexpected for those with liver transplants. It appears that in ovarian transplants to the liver, the restricted amount of liver in the path of drainage from the graft was insufficient to metabolize a significant proportion of the hormones released, as has also been found for hormone pellets (*18*). The average weight of the uterus in the animals of these 2 groups was 473 mg. In contrast, the uterus of 1 animal in which an intrarenal transplant was resorbed was 76 mg.

Table 1 presents data for 41 animals that maintained successful intrasplenic ovarian grafts. In 3 animals not included in the table, the graft had been replaced by scar tissue. When the animals were killed, 4 females

(†) were found to have either a remnant of ovarian tissue *in situ* or a grossly visible vascular adhesion connecting the graft to vessels outside the portal system (average uterine weight, 755 mg.). With the exception of these 7 rats, representing technical failures, vaginal smears of all other females were at first anestrous (A) in type. Thus, any ovarian hormones secreted by the grafts were being effectively inactivated in passage through the liver. In a number of instances, vaginal smears later showed periodic or persistent cornification (C). (Cornification was detected in S34 at the end of 2 months, S19 in 5 months, S17 in 6 months, S41 and 43 in 7 months, S27 and 33 in 8 months, S29 in 9 months, and S44 in 11 months.) It is concluded that in these animals ovarian steroids were elaborated in such large amounts that the capacity of the liver to destroy them was

TABLE 1.—*Data for rats with ovarian transplants in the spleen**

Rat and sex	Vaginal smear	Age trans. (*months*)	Final weight (*gm.*)	Uterine weight (*mg.*)	Diameter trans., *mm.* (Volume trans., *mm.*^{3})
S1 ♀	A	1	245	82	7×5×5 (88)
S2 ♀	A	1	208	80	8×6×5 (120)
S3 ♀	A	2	255	60	10×8×7 (280)
S4 ♂	—	2	286	—	7×7×4 (98)
S5 ♀	A/A	3	315	87	7×4×4 (56)
S7 ♀	A/A	3	220	107	10×6×5 (150)
S8 ♀	A/A	4	305	55	4×3×3 (18)
S10 ♀	A/A	4	282	83	8×6×6 (144)
S12 ♀ †	C/C	4	222	370	4×3×3 (18)
S13 ♀	A/A/A	5	275	65	7×4×4 (56)
S14 ♂	—	5	305	—	8×4×4 (64)
S15 ♀	A/A	5	250	160	10×10×9 (450)
S16 ♀	A/A	5½	225	97	7×5×5 (88)
S17 ♀	A/C	5½	350	206	12×10×10 (600)
S18 ♀	A/A/A/A	6	308	78	6×6×4 (72)
S19 ♀	A/A/C/C	6	284	290	12×12×9 (648)
S20 ♂	—	6	328	—	11×7×5 (193)
S21 ♀	A/A	6	300	52	14×10×10 (700)
S22 ♀	A/A/A	7	335	215	15×12×12 (1116)
S23 ♀	A/A	7	300	cast.	7×6×4 (84)
S24 ♀	A/A	7	260	cast.	6×6×6 (108)
S25 ♀ †	C/C/C/C	8	275	1750	2×2×2 (4)
S26 ♀	A/A/A/A	8	270	290	22×17×17 (1590)
S27 ♀	A/A/A/C	8	300	318	13×12×12 (936)
S28 ♀	A/A/A/A	8½	332	cast.	13×12×10 (780)
S29 ♀	A/A/C	10	—	enlarg.	20×19×15 (2850)
S30 ♀	A/A/A/A/A	10½	223	74	8×7×5 (140)
S31 ♀	A/A/A/A/A	10½	250	82	5×4×3 (30)
S32 ♀	A/A/A/A/A	10½	345	360	20×15×14 (2100)
S33 ♀	A/A/A/C/C	10½	265	170	14×12×10 (840)
S34 ♀	A/C/C/A/C	10½	250	130	14×12×12 (1008)
S35 ♀	A/A/A/A/A	11	294	130	20×17×17 (2890)
S36 ♀	A	11½	300	500	45×36×30 (24300)
S37 ♀ †	A/C/C/A/C	12	314	260	2×2×2 (8)
S38 ♀ †	C/C/C/A/C	12	312	640	5×4×4 (40)
S39 ♀	A/A/A/A/A	12	350	100	16×12×10 (960)
S40 ♀	A/A/A/A/A	12	275	155	20×14×14 (1960)
S41 ♀	A/C/C	14	332	390	12×11×10 (660)
S42 ♂	—	14	380	—	19×12×10 (1140)
S43 ♀	A/C/C	14	274	555	13×9×8 (468)
S44 ♀	A/A/C	14	257	220	14×12×12 (1008)

*Key: A=anestrous smear; C=cornified smear.
86, 9, and 11 are omitted because the transplant was replaced by a scar.
†Ovarian remnant *in situ* or adhesion between spleen and extraportal vessels.

exceeded. This conclusion is supported by the observation that in all of these animals, as in those with ovarian remnants or adhesions, the uteri were significantly larger than in the animals exhibiting no vaginal cornification. Cornification was observed only in rats in which uterine weight exceeded 125 mg., although it was not detected in all of those with larger uteri. The average weight of the uterus in 9 animals with grafts 1 to 5 months old was 87 mg.; in 8 animals with grafts 5½ to 8½ months, 194 mg.; in 11 animals with grafts 10 to 14 months, 253 mg.

Control transplants to the kidney and liver.—Grossly, the ovaries transplanted to the kidney were generally flattened beneath the renal capsule and appeared to consist of numerous follicles and corpora lutea. In 4 animals with transplants of 5 months' duration or longer, there was instead a large clear cyst, about 1 cm. in diameter, with a few opaque spots on its surface. The average calculated volume of the intrarenal transplants (except those that were cystic) was 24 mm.^{3}. The liver transplants were spherical and averaged 16 mm.^{3} in volume.

Histologically, the noncystic transplants were composed of tightly packed follicles and corpora lutea; almost no epithelioid interstitial tissue was found (fig. 1). As in normal ovaries (*2*), the granulosa cells of all follicles were intensely basophilic (fig. 1). The follicular thecal cells contained abundant lipide droplets (fig. 11), which displayed an intense Ashbel-Seligman carbonyl reaction (fig. 2) and also an intense Schultz cholesterol reaction. In the atretic follicles, lipide droplets were present also in the granulosa cells; these stained with Sudan black but did not give the other reactions. In healthy follicles, alkaline-phosphatase activity was limited to the theca; in atretic follicles it occurred in the granulosa cells as well (fig. 7). The follicular fluid was intensely stained by the periodic acid-Schiff technique (after saliva digestion); the reticulum of the theca stained moderately.

Newly formed corpora lutea had large basophilic lutein cells (fig. 1) with only sparse, small lipide droplets, which gave a carbonyl reaction (fig. 10) but contained no detectable cholesterol (fig. 9). Older corpora consisted of shrunken, eosinophilic cells that contained abundant lipide droplets giving both a strong cholesterol reaction and a carbonyl reaction (figs. 9 and 10). Young corpora displayed marked phosphatase activity in their peripheral connective tissue as well as in the walls of the vascular sinusoids, but little in the lutein cells; older ones had unreactive boundaries but a more intense reaction in the vascular stroma (figs. 7 and 8). The reticulum and blood-vessel walls were stained by the periodic acid-Schiff procedure.

The 4 intrarenal transplants that had become cystic consisted of 1 or more thin-walled sacs filled with flocculent precipitate. The walls were lined with a dense connective-tissue membrane containing many epithelioid cells. Scattered along their walls were small, normal-appearing follicles and typical corpora.

The uteri of all the animals with successful intrarenal and intrahepatic transplants evinced signs of hormonal stimulation (*cf.* fig. 37). The

epithelium was generally tall and occasionally showed squamous metaplasia, which is known to result from prolonged stimulation by estrogen (*15*). Eosinophils were generally abundant in the stroma and muscularis (*cf.* fig. 6), an occurrence that has also been found to be correlated with estrogenic stimulation (*16*). Alkaline-phosphatase activity was intense on the apical borders of the epithelial cells, in the capillary walls and in the eosinophils (*cf.* fig. 17). Mucous droplets, stained by the periodic acid-Schiff method after saliva or diastase digestion, were frequently found in the apices of the epithelial cells, particularly those deep in the glands.

By way of contrast, the uterus of that animal in which an intrarenal transplant was resorbed possessed an atrophied endometrium typical of the castrate state. Phosphatase activity, moreover, was intense throughout the epithelial cells instead of being limited to the apices (*19*) (*cf.* fig. 14). No mucus occurred in the cells.

Transplants to the spleen.—In the 4 cases in which remnants of ovarian tissue *in situ* or adhesions between the spleen and extraportal vessels were found (S12, 25, 37, 38), the transplant was small and strongly resembled the normal ovary, like the control transplants to kidney and spleen. Similarly, the uteri appeared stimulated (fig. 17).

Grossly, the successful transplants, in the first few months, appeared nodular as though composed of corpora lutea. Some contained a few follicles. After 4 to 6 months, they became more varied in gross appearance. Some had a lobular organization, some appeared homogeneous, a few contained one or more large fluid-filled cysts (*e.g.*, S21). When cut open, most appeared healthy throughout. Only a few of the largest contained necrotic, hemorrhagic areas in their centers (*e.g.*, S26, 36).

The grafts tended to enlarge with age, although there was marked variability in size. The average calculated volume of successful grafts aged 1 through 5 months (11 specimens) was 138 mm.^{3}, of those aged 5½ through 8½ months (12 specimens), 576 mm.^{3}, and of those aged 10 through 14 months (14 specimens), 2692 mm.^{3}. There was no apparent difference between the transplants in male and female hosts, in either gross or microscopic aspect. None of the grafts showed signs of metastasis.

Histologically, the successful grafts of 1-month duration contained only occasional follicles (some with ova) and consisted principally of corpora lutea and luteinizing follicles (fig. 12). The follicular walls were intensely basophilic. Most of the corpora were only moderately basophilic, suggesting that they were more than a few days old. Lipide droplets were extremely small and sparse in both the follicular thecal cells and in the lutein cells. The carbonyl reaction was intense in the droplets of thecal cells, in both normal and luteinizing follicles, but weak in the lutein cells. The Schultz cholesterol reaction was absent. The follicular fluid of either normal-appearing follicles or those undergoing luteinization was only weakly reactive to the periodic acid-Schiff test. In alkaline-phosphatase preparations, the theca of follicles was intensely reactive (fig. 13), and the granulosa cells also displayed some activity in luteinized follicles. The

outer border of the younger-appearing corpora was well delineated by phosphatase activity, and the vessels were also reactive. Most of the corpora, however, lacked an intensely reactive rim, another indication that they were not newly formed.

At 2 months, follicles were extremely rare, none being found with ova and almost all appearing partially luteinized. The follicular epithelium of luteinized follicles was moderately basophilic, but the corpora uniformly displayed little basophilia (fig. 15). Lipide droplets were small in thecal cells and in most corpora (fig. 19) and generally gave only a faint carbonyl reaction, except in the peripheral cells of some of the corpora. Cholesterol preparations were virtually negative. Residual follicular fluid in luteinizing follicles was only faintly stained by the periodic acid-Schiff method. Phosphatase activity was present in the thecas of the rare follicles. Although the corpora appeared bounded by strands of connective tissue, these rims lacked phosphatase activity, intense reactivity being limited to the walls of the sinusoids (figs. 16 and 18).

The uteri of the 3 females killed within the first 2 months after operation had the histologic appearance of the completely castrated animal, possessing low epithelium in which the phosphatase reaction was diffuse throughout the cytoplasm (fig. 14).

The 10 healthy transplants of 3 to 5½ months resembled each other closely. In eosin and methylene-blue preparations, the hyperplastic ovarian tissue was composed almost entirely of corpora (fig. 20), although occasional atretic follicles could be found. The peripheral cells of the corpora were larger than those in the center, and in the two 5-month specimens, these peripheral cells were more basophilic than were those more centrally situated. Lipide preparations showed that most of the corpora contained little or no stainable material in their centers, while the larger peripheral cells contained considerable amounts (fig. 23). Occasional corpora, however, contained large amounts uniformly distributed. The carbonyl and cholesterol reactions followed the same pattern. In phosphatase preparations, the disparity in cell size resulted in the center of the corpora appearing much more reactive than the periphery because of the crowding of sinusoids (figs. 21 and 22).

The uteri of some females in this group (S7, 15, 17) showed a taller epithelium than those of the earlier specimens and displayed some mitotic activity. The glands penetrated deeper into the stroma and were highly contorted. Eosinophils were abundant in the stroma. Phosphatase activity, however, occurred throughout the epithelial cells rather than being limited to the surface. Periodic acid-Schiff preparations were not made.

Between 6 and 8½ months, the transplants became much more heterogeneous in appearance. In most, 2 main histologic components were recognizable. Some areas consisted of luteal masses, usually showing signs of central necrosis. Histochemically, these resembled the luteal bodies previously described. Other areas resembled granulosa-cell tumors,

in which most of the cells were small and quite basophilic; these masses of cells were surrounded and invaded by strands of larger, more eosinophilic, spindle-shaped thecal cells (figs. 3 and 4). The areas of small basophilic cells were avascular, while the strands of eosinophilic cells were vascularized. In some portions, many of the small cells were pyknotic, while in others mitoses were abundant (fig. 25). In some specimens, these areas of granulosa cells contained fluid-filled cavities (figs. 3 and 24). The small granulosa-like cells contained only traces of lipides (figs. 26 and 29) and these were essentially negative for both carbonyl groups (figs. 5 and 27) and cholesterol (fig. 28). These cells were also largely free of phosphatase activity (fig. 24). The surrounding spindle-shaped cells, on the other hand, contained numerous small lipide droplets that reacted for both carbonyl groups and cholesterol (figs. 5 and 26 to 29). The walls of the sinusoids, and possibly the spindle-shaped cells as well, possessed intense phosphatase activity (fig. 24). The female hosts in this group of animals all showed highly stimulated uteri (*cf.* fig. 6).

Two of the transplants of this age group differed markedly from those described in the preceding paragraph. S21 consisted of a cyst, the walls of which appeared to be a disorganized, unusually basophilic luteoma. Many of the cells contained large quantities of lipide, while others were empty. The uterus appeared castrate. The other exceptional tumor, S26, displayed 2 types of tissue, a small portion consisting of aggregations of typical luteal tissue, and a large portion composed of small stellate cells embedded in considerable quantities of amorphous matrix (fig. 30). The latter region exhibited an extraordinary degree of phosphatase activity (fig. 31), but lipide was very sparse (fig. 32) and entirely negative to both the carbonyl and cholesterol tests. The abundant interstitial ground substance was faintly stained by the periodic acid-Schiff method. The uterus of this animal showed marked signs of estrogenic stimulation.

In the group of transplants of 10 to 14 months' duration, most consisted predominantly of masses of luteal tissue (fig. 38). Five, however, appeared to be principally granulosa-cell tumors (S29, 32, 33, 36, 43) (fig. 36). It was rare to find a transplant uniform in its composition. Some luteal tissue was present in the predominantly granulosa-cell tumors, and conversely, some areas of granulosa cells were present in those transplants which appeared grossly to be luteomas (fig. 35). In addition, several of the transplants contained large areas in which whorls of epithelioid cells were embedded in fibrous connective tissue (figs. 33 and 34).

In tumors of this age group, the small cells in granulosa-cell areas were generally quite basophilic, the corpora were only slightly basophilic, whereas the epithelioid cells arranged in whorls were distinctly eosinophilic (fig. 33). Mitoses were common in the small granulosa cells (fig. 34). Lipide was generally, though not invariably, sparse in the luteal areas (fig. 38). It was present in moderate concentrations in the thecal strands of the granulosa-cell tumors and, in lesser concentration, in the granulosa

TABLE 2.—*General histochemical characteristics of ovarian grafts (for exceptions see text)**

Structure	Basophilia	Alkaline phosphatase	PAS	Lipides		
				Sudan	Carbonyl	Cholesterol
Intrarenal and Intrahepatic Grafts						
Follicles (normal)	+++ (g c)	+++ (t c and s)	++ (f f) + (s)	++	++	++ (t c)
Follicles (atretic)	++ (g c)	++ (g c) +++ (t c and s)	++ (f f) + (s)	+ ++	0 ++	0 (g c) ++ (t c)
Corpora lutea (new)	++ (l c)	++ (rim and s)	+ (s)	+	++	0 (l c)
Corpora lutea (old)	(+) (l c)	++ (s)	+ (s)	++	++	++ (l c)
Intrasplenic Grafts						
1. With vascular adhesions, like intrarenal and intrahepatic grafts.						
2. Successful grafts, without vascular adhesions.						
a. 1–5½ mos.						
Luteal bodies	(+) (l c)	++ (s)	+ (s)	+	++	0 (l c)
Follicles (atretic)	++ (g c)	++ (g c) +++ (t c and s)	+ (f f) + (s)	+ ++	+ ++	0 (g c) ++ (t c)
b. 6–8½ mos.						
Luteal bodies, like those in group *a*.						
G c tumors	++ (g c)	++ (t c and s)	+ (f f) + (s)	(+) ++	0 ++	0 (g c) ++ (t c)
c. 10–14 mos.						
G c tumors, like those in group *b*.						
Luteal bodies, some like those in groups *a* and *b*, *or*	0	++ (s)	+ (s)	+++	+	+++ (l c)
Epithelial whorls	0	+ (s)	0	++	++	++

*Key: g c = granulosa cells; t c = thecal cells; s = stroma; l c = lutein cells; f f = follicular fluid; (+) trace; + small amount; ++ moderate amount; +++ large amount.

cells proper. The cells in the whorls were also generally fatty. The lipides of the luteal cells and of the thecal cells in the strands (but not of the granulosa cells) were positive for carbonyls. The cholesterol reaction tended to parallel the sudanophilia. The granulosa-cell tumors contained spaces filled with a precipitate, which in some cases stained intensely by the periodic acid-Schiff technique (fig. 36), in others only weakly. In the luteal areas, alkaline-phosphatase activity was limited to the sinusoid walls (fig. 35). In the granulosa-cell tumors, activity occurred principally in the strands of thecal cells. The zones of connective tissue containing epithelial whorls were intensely reactive.

Uteri of some females in this age group were nearly castrate in appearance (fig. 39), whereas others were stimulated (fig. 37). In general, stimulated uteri were associated with transplants that consisted largely of granulosa-cell areas.

A summary of the histochemical characteristics of the grafts is presented in table 2.

Re-establishing connection with systemic blood vessels.—An experiment designed to provide further evidence for the production of estrogens by the tumors was performed on a single animal, S28, at 8½ months, at which time a biopsy revealed a disorganized mass of luteal tissue, the vaginal smear was anestrous, and the uterus was essentially castrate in appearance. The cut edge of the tumor was then sutured to the body wall. Subsequent to the operation, the vaginal smear consisted of cornified cells only, indicating release of large amounts of estrogen into the general circulation. Three weeks after anastomosis, the abdomen was reopened. The transplant appeared softened, and sections of a uterine biopsy taken at this time showed marked stimulation of the endometrium. Ten days later the animal was killed. The transplant now consisted of a large cyst filled with a straw-colored fluid. Histologically, the cyst had a fibrous wall without a detectable epithelial lining. Within the connective-tissue wall, there were occasional patches of vacuolated spindle-shaped cells. The uterus still showed histologic signs of marked estrogen stimulation.

Discussion

A histophysiologic study of the normal rat ovary during the estrous cycle (*2*) led to the following conclusions that bear on the present study. (*a*) Lipide droplets in the cells of the theca interna, interstitial tissue, and corpora lutea contain precursors of the ovarian steroid hormones. (*b*) When one of these tissues is secreting actively, the droplets are small and deficient in cholesterol but rich in secondary carbonyl lipides.[7] Contrariwise, when that tissue is not actively secreting, the droplets enlarge and cholesterol accumulates, but the concentration of carbonyl compounds eventually declines. (*c*) Correlatively, the cells are large when the tissue

[7] It was formerly concluded that these lipide carbonyl compounds represented ketosteroids (*8,7,17*). More recent experimental evidence (*20*), however, indicates that they are fatty aldehydes produced by the autoxidation of highly unsaturated fatty acid moieties during fixation of the tissue. However, Karnovsky and Deane (*20*) adduced some evidence that the concentration of unsaturated fatty acids may be directly related to the secretory activity of steroid-producing organs.

is actively secreting, small when it is inactive. (*d*) Alkaline phosphatase is abundant in the blood vessels adjacent to actively secreting ovarian parenchymal cells.

In the present experiment, most of the intrarenal and intrahepatic transplants, as well as the intrasplenic transplants that developed adhesions, bore a marked similarity to normal ovaries, except that they showed a paucity of interstitial tissue. Interstitial tissue presumably arises from atretic follicles (*2,21*); hence, its deficiency in these transplants in which atretic follicles were abundant remains baffling.

The early intrasplenic transplants of our series (1 to 5½ months) consisted predominantly of luteal bodies, and the lutein cells appeared highly active, inasmuch as they were large and their lipide droplets were small and positive for carbonyl groups but deficient in cholesterol. Since the females with such tumors displayed no vaginal or uterine stimulation, it may be assumed that most of the hormones secreted were inactivated by the liver. Kullander (*22*), however, has adduced evidence that significant quantities of progesterone may escape inactivation.

Most of the tumors of intermediate age (6 to 8½ months) consisted primarily of granulosa cells. Secretion of estrogen in excess of the liver's capacity to inactivate was suggested by the vaginal and uterine stimulation. In another experiment, not reported here, it was found that the pituitaries of animals possessing tumors of this sort lacked the vacuolated basophils or "castration" cells that develop in the absence of ovarian hormones (*23*). In such granulosa-cell tumors, however, it was not the granulosa cells proper that evinced signs of steroid-hormone secretion, but rather it was the surrounding and invading strands of thecal cells. As in normal follicles, these cells were large and contained numerous small lipide droplets, and the accompanying vascular sinusoids displayed intense alkaline-phosphatase activity. Usually, the granulosa cells proper were extremely small and devoid of free lipide. In some instances they contained large droplets, as they do in atretic follicles, where lipide accumulation is a stigma of degeneration rather than an evidence of secretion. Another cogent argument against any endocrine function for the granulosa cells lies in the absence of adjacent blood vessels. The overgrowth of granulosa cells in these tumors probably results from 2 stimuli: namely, pituitary FSH and the locally produced estrogen (*24*). The belief that the thecal elements in granulosa-cell tumors are probably responsible for estrogen secretion has also been voiced by McKay, Robinson, and Hertig (*25*), despite the prevailing opinion (*26–28*) that it is the granulosa cells proper that are responsible for the feminizing action of these tumors.

In this connection, it is interesting to speculate on the conversion of a luteoma to a granulosa-cell tumor. Since no new follicles were detected in the older transplants nor any signs of proliferation from the germinal epithelium, we can only conclude that granulosa lutein cells dedifferentiated (*see also 4,14*). Not only did the cells regain the small size and cytoplasmic basophilia characteristic of typical granulosa cells but also their ability to undergo mitosis and to secrete a mucoid follicular fluid.

A modulation in this direction has never been described for the ovary *in situ*, but it might be responsible for many of the confusing features of clinical ovarian tumors. Certainly none of the observations reported here support the clinical opinion (*26,27*) that granulosa cells arise from thecal or stromal elements.

In our series, the transplants of more than 9 months' duration again appeared to be, for the most part, luteomas. We cannot know whether or not these had reverted from granulosa-cell tumors. In these tumors the luteal bodies were more varied in appearance than they were in the 1-to-5-month group. In a single transplant, some corpora might be composed of large cells exhibiting a paucity of small droplets (presumably active), whereas others contained smaller cells heavily laden with lipide that displayed abundant cholesterol but little carbonyl lipide (presumably inactive). Many of these older tumors also contained large areas of granulosa cells, illustrating a local variability in tissue response to prolonged gonadotrophin stimulation.

A final cellular arrangement that bears mention is the whorls of epithelioid cells observed in many of the older transplants. These probably represented isolated nests of thecal cells. Since they generally appeared large and lipide-filled, it may be assumed that they were secretory, but, of course, the nature of their product could not be ascertained.

In this series, 1 transplant was encountered whose organization defied classification (S26). The structure was composed almost entirely of stellate cells embedded in a mucoid ground substance, thus resembling mesenchyma. The most unusual aspect of this tumor was its extraordinary concentration of alkaline phosphatase. The small stellate cells contained lipide droplets, and the tumor was apparently secreting estrogen in large enough quantity to exceed the liver's inactivating capacity, as indicated by the stimulation of the uterus.

Endocrine considerations.—Brief comment on the endocrine imbalance that may have produced the changes observed in the structure of the intrasplenic ovarian transplants appears warranted. Greep and Chester Jones (*7*) deduced from a variety of experimental data that two patterns of gonadotrophin imbalance may develop in gonadectomized animals. In one type, luteotrophin (LTH) gains dominance, in which case the output of both FSH and LH is low. It has been shown repeatedly that a high LTH/LH ratio maintains active corpora lutea. It seems logical to assume that this pattern must prevail in the gonadectomized animal for the first few months after the transplantation of an ovary to the spleen. Parenthetically, it is interesting to note that such luteomas developed in males as well as in females, thus providing evidence for the secretion of LTH by the male pituitary. The usual gonadotrophin pattern for the long-term castrate, however, is high FSH, low LH, and low LTH output (*7*). Since FSH stimulates granulosa-cell proliferation, it may be assumed that this pattern had supervened in those animals which secondarily developed granulosa-cell tumors (*29*).

Another intriguing question arises from the fact that many of these

tumors, especially those consisting predominantly of granulosa cells, apparently produced such large amounts of estrogen that much escaped inactivation in the liver (*see also 4,9,14*), since in these animals vaginal cornification and uterine stimulation did develop in the absence of vascular adhesions. One might expect that such a development would be self-regulatory, in the sense that the estrogen would depress FSH and LH output (*30*) and thus permit LTH to become dominant again. Indeed, this course may have occurred in some of our oldest animals that exhibited tumors consisting almost entirely of luteal tissue. Many animals, however, displayed persistent vaginal stimulation for a period of months. It therefore appears that once a particular imbalance of gonadotrophins has become established, its correction is very difficult.

Cystic ovaries can be produced by the administration of large quantities of FSH (*31*). Such an augmented supply of FSH may account for the development of cysts in some of the older intrarenal transplants and in occasional intrasplenic transplants. A cyst also developed in one animal with a successful intrasplenic transplant in which the spleen was deliberately anastomosed to the body wall. These observations serve to bear out the above deduction that once an imbalance of pituitary gonadotrophin output has developed, a return to normal is not easily produced. Why such imbalance supervened in some animals with intrarenal transplants deserves more study (*see also 9*).[8]

Summary and Conclusions

The development, morphology, and histochemistry were studied in ovarian transplants to the spleen, kidney, or liver of gonadectomized rats. In female rats, the secretory activity of the transplants was assessed by vaginal smears and by uterine weight and histology.

The intrarenal and intrahepatic transplants, as well as those intrasplenic transplants to which an extraportal vascular connection developed, remained small, contained normal follicles and corpora lutea, and caused typical stimulation of the vagina and uterus.

Successful intrasplenic transplants grew progressively larger. During the first 5 months, they consisted principally of corpora lutea, which gradually became disorganized. In the 6-to-9-month group, most contained large areas resembling granulosa-cell tumors. In the 10-to-14-month group, they contained luteal tissue, areas of granulosa cells, and also whorls of epithelioid cells embedded in connective tissue. None of the tumors became invasive or metastasized.

The histochemical procedures employed assisted greatly in the identification of the tissues. Furthermore, they contributed to the histophysiologic evidence of active secretion by the young luteomas and by the thecal cells in the granulosa-cell tumors. The estrogen output of granulosa-cell tumors always exceeded the inactivating capacity of the liver, as indicated by vaginal and uterine stimulation.

[8] In a paper found after this went to press, Fels (*32*) has reported that, after 1 year, many intrarenal and all intrahepatic ovarian transplants in gonadectomized rats became tumorous.

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Plate 30

Intrarenal and intrasplenic transplants

Figure 1.—One-month intrarenal transplant. The transplant consists of normally basophilic follicles and of corpora lutea of varying age. Eosin and methylene blue, *p*H 5.13. × 90

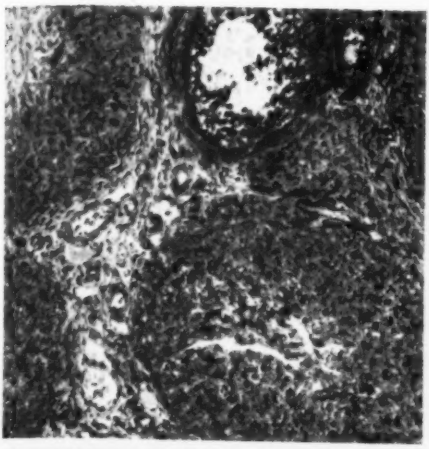
Figure 2.—Same transplant. The lipide droplets in the thecal cells (*left*) and lutein cells (*right*) react positively. Ashbel and Seligman carbonyl method. × 200

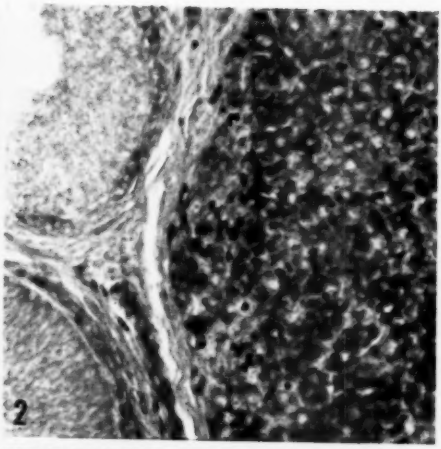
Figure 3.—Seven-month intrasplenic transplant, S22. The transplant consists of lobules of small, highly basophilic granulosa cells, surrounded and invaded by strands of larger, eosinophilic thecal cells. Eosin and methylene blue. × 90

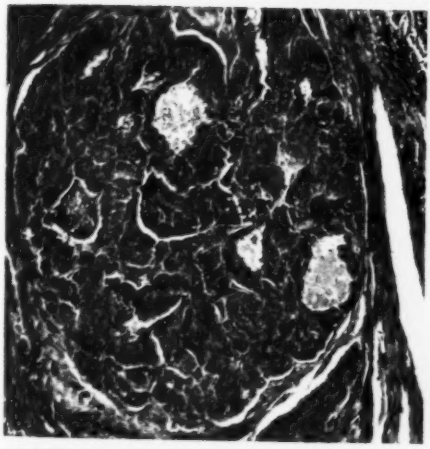
Figure 4.—Same preparation. The strands of thecal cells are conspicuous. × 200

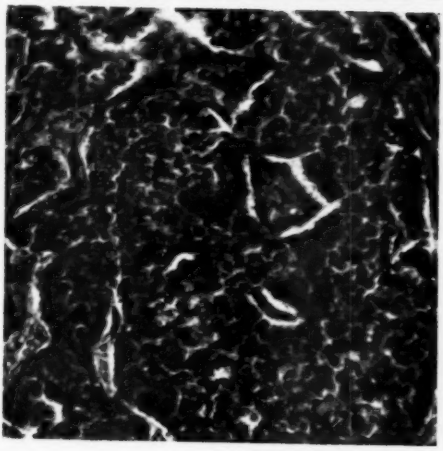
Figure 5.—Ten and one-half month intrasplenic transplant, S33. Reactive lipides are confined almost entirely to the thecal cells. *Cf.* figure 24. Ashbel and Seligman carbonyl method. × 200

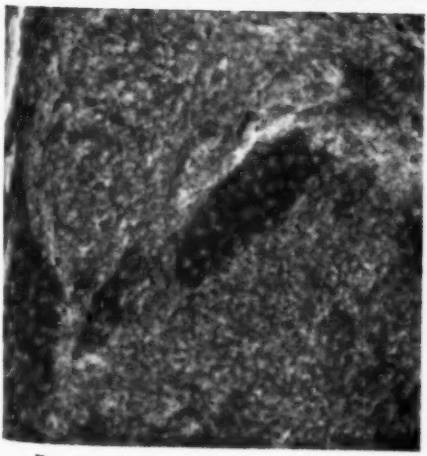
Figure 6.—Uterus of animal with 8-month intrasplenic transplant, S27. In this animal with a granulosa-cell tumor, the uterine epithelium is tall and eosinophils are abundant in the underlying stroma. Eosin and methylene blue. × 200

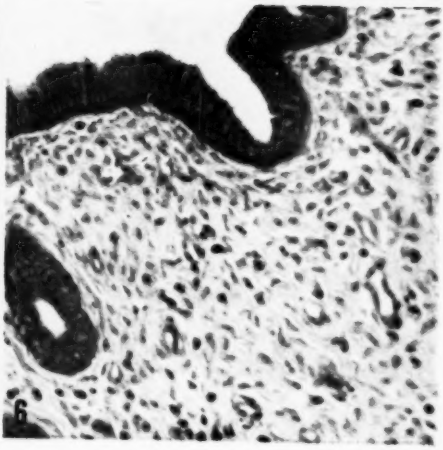












Deane and Fawcett

PLATE 31

Intrarenal transplants

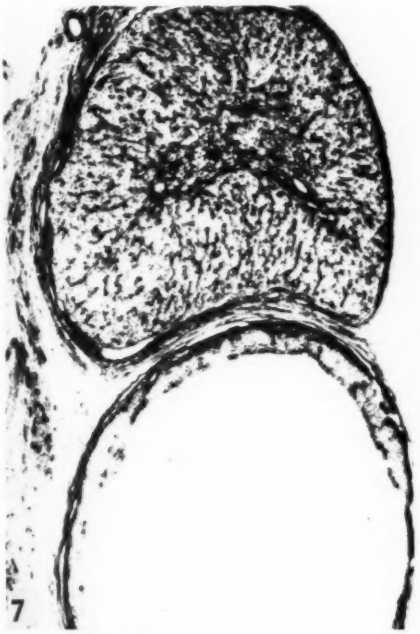
FIGURE 7.—Six-month transplant. *Above*, a recent corpus luteum; *below*, a luteinizing follicle. Note that alkaline-phosphatase activity is particularly high in the theca interna of both the corpus and follicle, and in the walls of sinusoids. Incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters. × 50

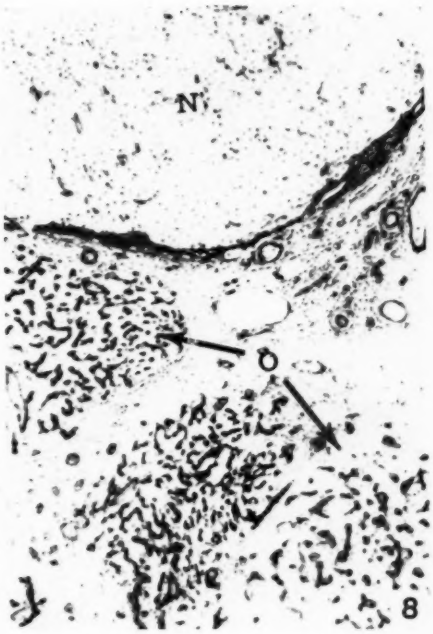
FIGURE 8.—Eight-month transplant. The recently formed corpus luteum (N) shows an intensely reactive rim and weakly reactive sinusoids, whereas the retrogressing corpora (O) lack reactive rims but display greater activity in the sinusoidal walls. Incubated in glycerophosphate, *p*H 9.5, 5 hours, green and yellow filters. × 100

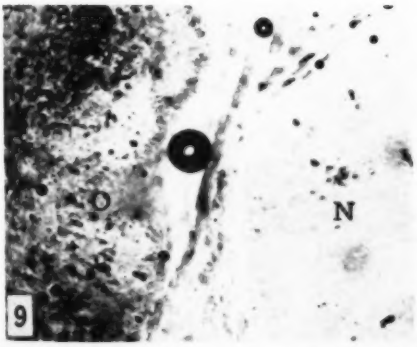
FIGURE 9.—Same transplant. The recently formed corpus luteum (N) contains no detectable cholesterol, whereas the old corpus luteum (O) contains abundant cholesterol. Schultz cholesterol reaction, red filter. × 90

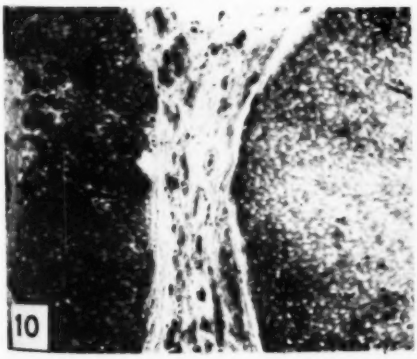
FIGURE 10.—Same transplant. The sparse lipide droplets in the newly formed corpus to the *right* display a moderate carbonyl reaction; the larger droplets in the older corpus to the *left* react intensely. Ashbel and Seligman carbonyl reaction, red filter. × 90

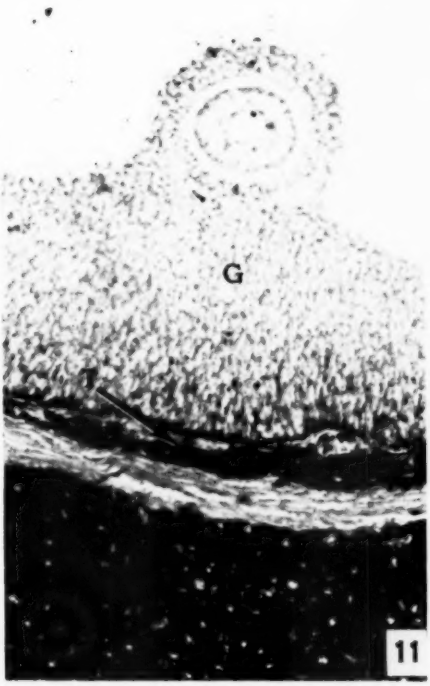
FIGURE 11.—Five-month transplant. Lipide droplets are abundant in the cells of the theca interna (T) and in the cells of the old corpus luteum (*below*). Some lipide droplets appear in the basal cells of the granulosa (G), indicating that the follicle is atretic. Sudan black B, 7 minutes, blue filter. × 200











Deane and Fawcett

395335—56——5

PLATE 32

Early intrasplenic transplants

FIGURE 12.—One-month transplant, S2. The transplant is composed principally of moderately basophilic corpora lutea; one atretic follicle above. *Cf.* figure 1. Eosin and methylene blue, yellow and orange filters. × 50

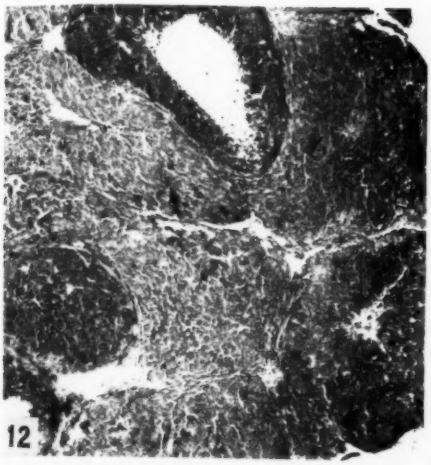
FIGURE 13.—Same transplant. The theca of the follicle reacts intensely, as do the walls of the sinusoids and the rim of the large, newly formed corpus luteum (N). The rest of the corpora (O) lack reactive rims. *Cf.* figure 8. Incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters. × 50

FIGURE 14.—Uterus of animal with 4-month transplant, S10. The epithelium is low. The entire cytoplasm of the epithelial cells and the walls of the sinusoids are intensely reactive. *Cf.* figure 39. Incubated in glycerophosphate, *p*H 9.5, 5 hours, green and yellow filters. × 50

FIGURE 15.—Two-month transplant, S4. The section consists of numerous weakly basophilic corpora lutea. Prepared like figure 12.

FIGURE 16.—Two-month transplant, S3. None of the corpora display rims reactive for alkaline phosphatase. Prepared like figure 13.

FIGURE 17.—Uterus of animal with 4-month transplant, from which there was a vascular adhesion outside of the portal system, S12. *Note* the taller epithelium, in which alkaline phosphatase is limited to the apices of the cells. Tissue eosinophils (*arrow*) are intensely reactive. Prepared like figure 14.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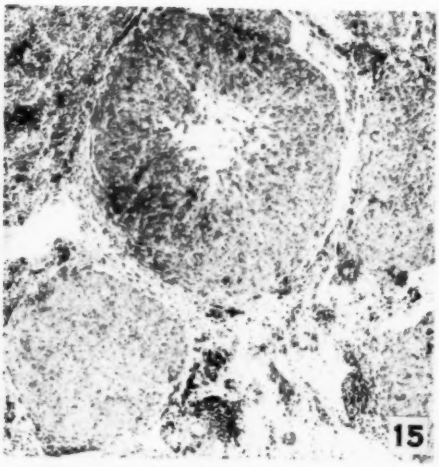
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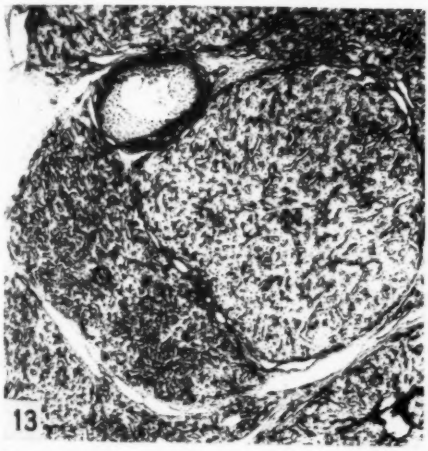
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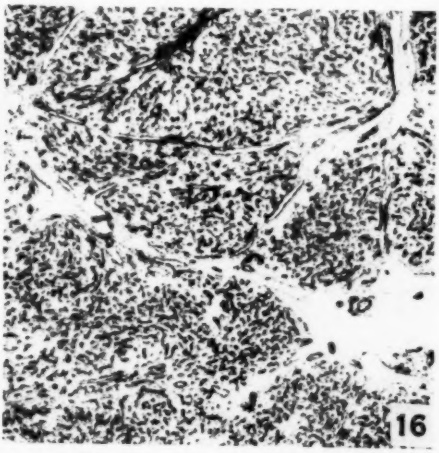
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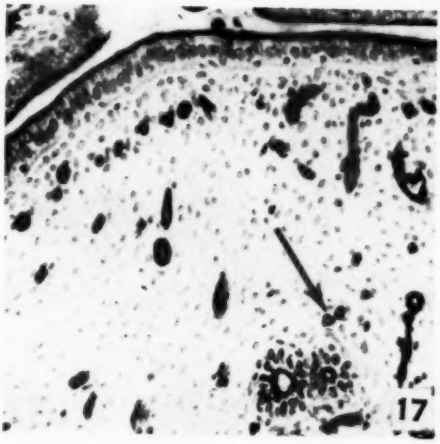
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PLATE 33

Intrasplenic transplants, 2 to 5 months

FIGURE 18.—Two-month transplant, S3. Principal sites of phosphatase activity are the walls of sinusoids. The relatively new corpus luteum on the *right* possesses an active rim as well. Incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters. × 200

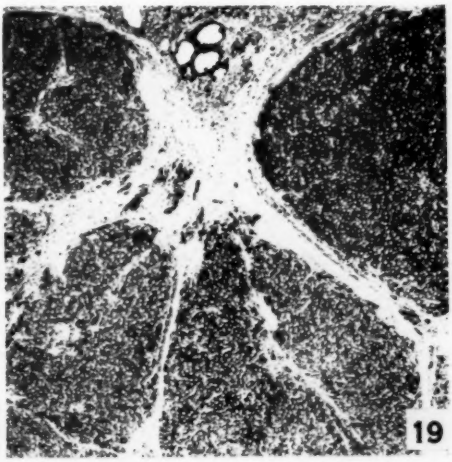
FIGURE 19.—Two-month transplant, S4. All of the lutein cells contain abundant, small lipide droplets. Sudan black B, 7 minutes, green and yellow filters. × 50

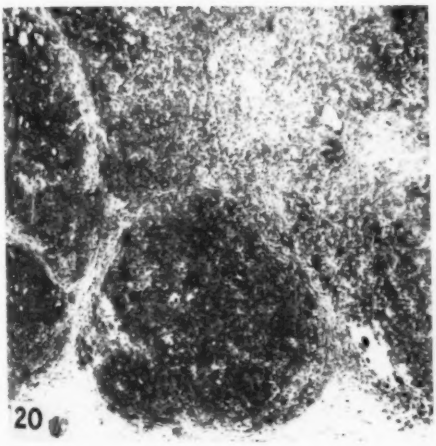
FIGURE 20.—Five-month transplant, S15. The transplant appears to be a somewhat disorganized luteoma. Eosin and methylene blue, yellow and orange filters. × 50

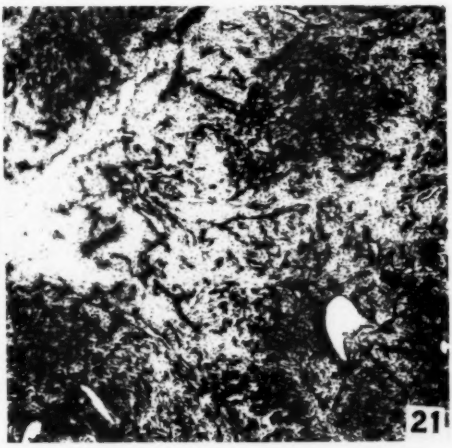
FIGURE 21.—Five-month transplant, S15. The cells at the centers of the corpora are shrunken so that the reactive sinusoids are close together. The corpora have completely lost their reactive boundaries. Prepared like figure 18.

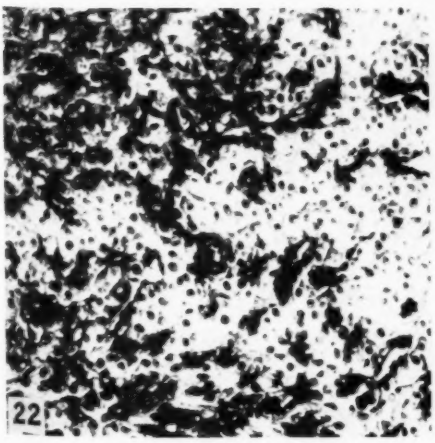
FIGURE 22.—Four-month transplant, S10. Junction of 2 corpora. The cells lying in the centers of the corpora are small, while those at the periphery are large. No phosphatase-reactive rims occur. Prepared like figure 18.

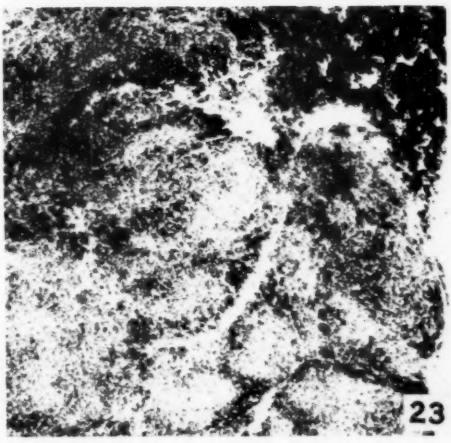
FIGURE 23.—Five-month transplant, S15. In most of the corpora, lipides have virtually disappeared from the central cells. Prepared like figure 19.











395335—56——6

PLATE 34

Granulosa-cell tumors, 7 to 10½ months

FIGURE 24.—Ten and one-half-month transplant, S33. Phosphatase activity is restricted to the thecal strands (T) and is absent from the granulosa cells (G). Incubated in yeast nucleic acid, *p*H 9.5, green and yellow filters. × 200

FIGURE 25.—Ten and one-half month transplant, S32. Cell division (M) characterizes the basophilic granulosa cells. *Note* strands of larger, eosinophilic thecal cells (T) deep among the granulosa cells. *Cf.* figure 4. Eosin and methylene blue, yellow and orange filters. × 450

FIGURE 26.—Eight-month transplant, S27. Lipide droplets are abundant in the thecal cells, sparse in the granulosa cells. Sudan black B, 7 minutes, blue filter. × 200

FIGURE 27.—Same transplant. Only the thecal lipides are carbonyl-reactive. Ashbel-Seligman carbonyl reaction, red filter. × 200

FIGURE 28.—Seven-month transplant, S22. The lipides of the thecal strands contain abundant cholesterol. Schultz cholesterol reaction, red filter. × 90

FIGURE 29.—Same transplant. Lipide droplets are abundant but small in the large thecal cells, sparse but large in the smaller granulosa cells. *Cf.* follicle, figure 11. Prepared like figure 26. × 90

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T
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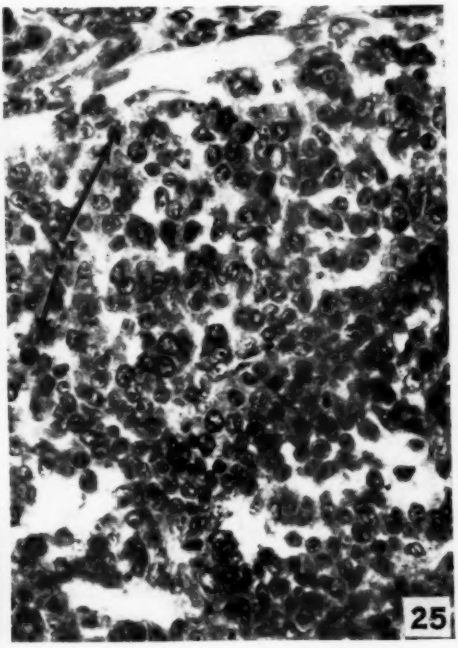
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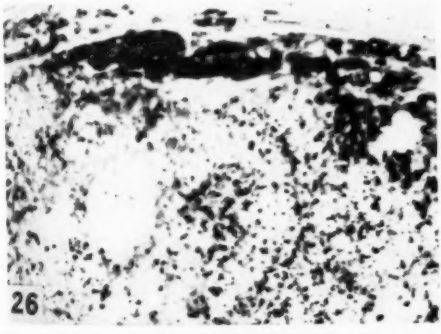
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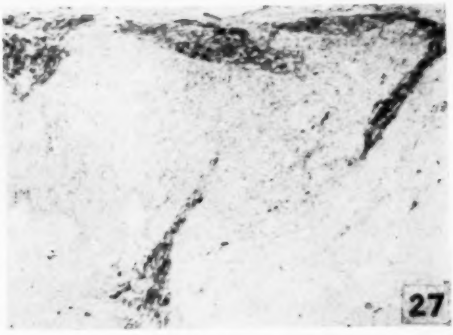
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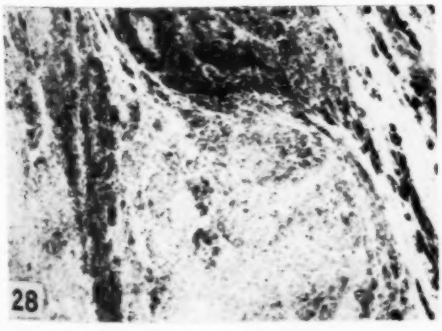
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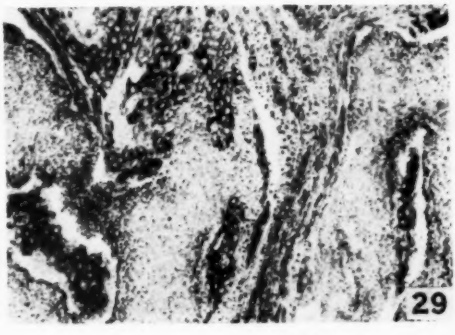
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Plate 35

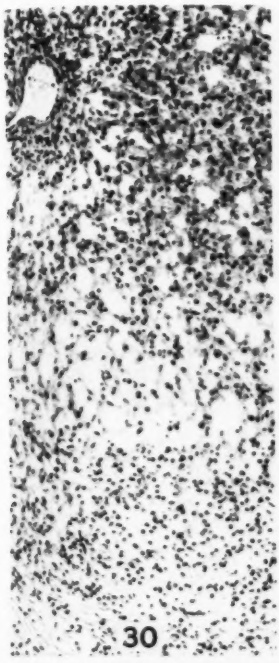
Intrasplenic transplants, 8 to 12 months

Figures 30 to 32.—Eight-month transplant, S26. Most of the tumor is composed of small, stellate shaped cells embedded in what appears to be a mucoid (periodic acid-Schiff-positive) matrix. *Note* the intense alkaline-phosphatase reaction of this tissue in figure 31 (normal luteal tissue *above*). Lipide droplets are sparse (fig. 32). Figure 30, Zenker-acetic, hematoxylin and eosin, green and orange filters; figure 31, incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters; figure 32, Sudan black B, 7 minutes, blue filter. All × 100

Figure 33.—Twelve-month transplant, S40. Slightly basophilic luteal tissue *above* and whorls of eosinophilic, spindle-shaped cells (W) embedded in connective tissue *below*. Eosin and methylene blue, yellow and orange filters. × 200

Figure 34.—Ten and one-half month transplant, S32. Area of granulosa cells *above* (*note* mitoses), whorls of spindle-shaped cells *below*. Prepared like figure 33.

Figure 35.—Ten-month transplant, S29. This illustrates the heterogeneity of many older transplants, showing corpora (C) of different types and granulosa-cell tumor (G). In this animal, the blood vessels were injected with India ink and the sinusoids are thereby blackened. Incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters. × 20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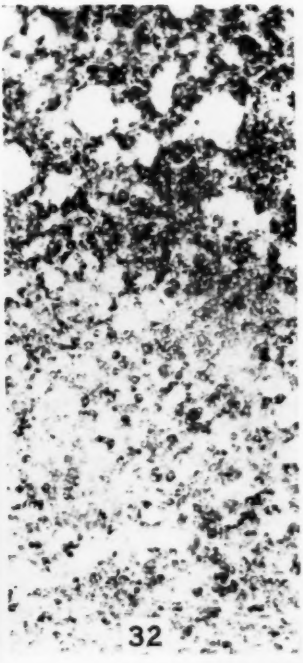
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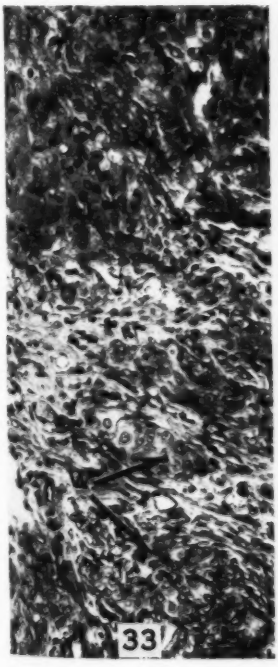
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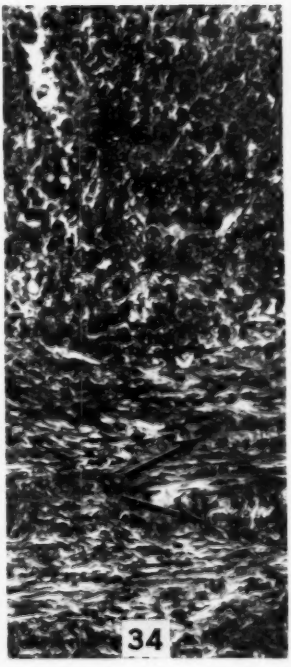
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PLATE 36

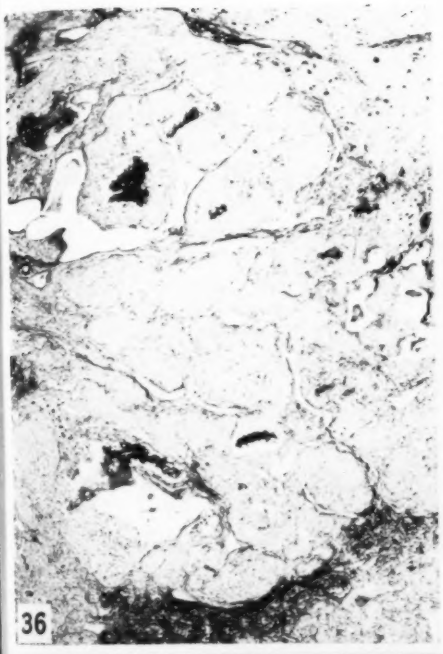
Intrasplenic transplants, 11½ to 12 months

FIGURE 36.—Eleven and one-half month transplant, S36. The follicular fluid in this region of granulosa-cell tumor reacts intensely for carbohydrate. Periodic acid-Schiff reaction, green filter. × 50

FIGURE 37.—Uterus of same animal. The uterus is markedly stimulated; one gland is filled with pus. Eosin and methylene blue, yellow and orange filters. × 50

FIGURE 38.—Twelve-month transplant, S39. Some of the luteal bodies contain sparse lipide droplets where others are heavily laden. Sudan black B, 7 minutes, green and yellow filters. × 20

FIGURE 39.—Uterus of same animal. The uterus appears relatively unstimulated. Incubated in yeast nucleic acid, *p*H 9.5, 5 hours, green and yellow filters. × 50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Observations on the Morphology and Histochemistry of the Mouse Pituitary Implanted in the Anterior Eye Chamber [1]

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Previous investigations from this laboratory have indicated that the transplanted mouse pituitary is capable of maintaining thyroidal metabolism of I^{131} in the host but is incapable of maintaining the size of any of the target endocrine glands, including the thyroid, above that of the hypophysectomized state (*1*). It was hoped that cytologic and histochemical study of such functionally limited heterotopic glands might reveal some information concerning the morphologic basis of their peculiar physiologic activity. The present study makes use of the periodic acid-Schiff technique, which demonstrates histochemically the glycoprotein of the basophils; a selective trichrome stain; and a toluidine blue-ribonuclease method for identifying ribonucleic acid. The mice used were of the same genetic constitution as those in the previous functional studies and the implants were prepared in like manner. Whole mouse pituitaries were implanted, and observations made of all 4 parts of the gland. The implants were taken for examination at intervals beginning 1 day after implantation and extending to 14 months so as to document the sequence of changes taking place in the implanted tissues.

Morphologic studies of transplanted pituitaries in rats (*2–8*), guinea pigs (*9*), and mice (*10*) have been reported. However, the present study combined the utilization of mice and observation of all lobes of the gland with the differential and histochemical techniques employed, and in addition surveyed the implants at early and frequent intervals following transplantation.

Methods

All animals used in the study were F_1 derivatives of BALB/c × C3H mice. Whole pituitary glands were dissected from newborn mice of either sex and implanted, 1 per host, into the anterior eye chamber of 42 normal

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[4] The authors are indebted to Dr. Clifford Grobstein for providing the mice and performing implantations, and to Mr. Robert R. Padgett and Mr. John T. McGuire for taking the photographs.
[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

395335—56——7

2-month-old female mice by a technique previously described (*11*). Host mice were maintained for intervals ranging from 1 day to 14 months after implantation. Animals were killed at the times following implantation listed in table 1. At each interval 2 to 7 mice were killed with ether, and the graft-bearing eye was removed and fixed in buffered mercuric chloride-formol (*12*). For comparison, pituitaries were also secured from normal mice of both sexes at various intervals from birth to 3 months. Serial paraffin sections, 3 and 5 μ, were prepared of each implant *in situ*.

The majority of sections were stained by the periodic acid-Schiff method (PAS), which demonstrates the sites of glycoprotein, or by a modified Mallory triple stain (*13*), which is selective for demonstrating pituitary cell types. Control PAS slides were exposed to saliva for 15 minutes at room temperature prior to staining. Staining for ribonucleic acid was performed with toluidine blue in acetate buffer at *p*H 4.5 or 5.0. Before staining, 1 of a pair of adjacent slides was incubated 1 to 1½ hours at 37° C. with 100 μg. of ribonuclease in 0.5 ml. distilled water. During this period the adjacent control sections were allowed to stand in distilled water at the same temperature. As a certain amount of extraction of the stain takes place during the dehydration in alcohol, care was taken to carry each slide simultaneously with its control through all parts of the procedure.

The volumetric measurements of the pituitary implants, given in table 2, were calculated by means of planimetry of serial sections.

Observations

General Morphology of Anterior Lobes of Implanted Pituitaries

One to 8 days after implantation.—All the 12 transplanted pituitaries examined at 1 to 8 days following implantation showed varying but sometimes marked degrees of degeneration and necrosis. One of the 8-day implants showed intact cells and little necrosis. The sinusoids of the anterior lobe were often distended and engorged with blood. Cellular and amorphous debris were trapped in residual lumina of Rathke's pouch and in scattered necrotic areas within the anterior lobe. In both anterior and intermediate lobes nuclei were frequently predominantly pyknotic or hyperchromophilic. The cytoplasm of the epithelial cells tended to be indistinct, cell boundaries undelineated, and the tissue appeared somewhat edematous. In the anterior lobes of most of the implants, centrally placed necrotic tissue and extravasated erythrocytes could be seen surrounded by an irregular cortex of relatively healthy cells (fig. 1). Within the debris, chromophils in which no stainable nuclear material remained could readily be identified, but the cytoplasm was intact and could be selectively or specifically stained (fig. 2).

One day after implantation, small blood vessels appeared to enter the implant from the iris at points of contact. However, judging from the extravasated blood in the tissue and the dilatation of the anterior-lobe sinusoids, which were packed with erythrocytes, revascularization was

not well established until approximately the 8th day. It must be pointed out that in all but 1 of the implants of 1 to 8 days, *varying* amounts of intact, healthy epithelial tissue could be seen (fig. 3), but their intact state could not be correlated with the length of time after implantation. One of the 3-day implants looked completely necrotic throughout the serial sections, and no attachment to the iris could be found.

The tissue degeneration in the initial stages after implantation was apparently not due to mechanical injury to the pituitaries by the dissection procedure prior to implantation. Three pituitaries from newborn animals were dissected as for implantation, allowed to stand in Tyrode's fluid 20 to 60 minutes, then fixed and prepared for histologic examination. Except for a thin marginal rim, 2 or 3 cells thick, showing histologic signs of damage, these pituitaries were not detectably different from 4 other pituitaries of newborn mice that were fixed *in situ* before dissection and histologic preparation.

Eleven days or more after implantation.—At 11 or more days, the epithelial tissue was intact, cell boundaries were distinct, and the vascular pattern was more or less normal with the exception of 1 unusually small 15-day implant. The only indication, in the remainder of the series, of the degeneration of tissue that occurred during the first week was the presence of rather large cavities within the epithelial tissues containing variable amounts of blood, and, in the 11- to 15-day groups, some debris as well. The debris was apparently absorbed or phagocytosed progressively with time and was not seen after 15 days.

Between the day of implantation and day 15 the implanted tissue grew (table 2) in spite of the necrosis that occurred in the early period. Mitoses were frequently seen in all implanted anterior lobes after the 8th day but were quite infrequent before this time.

Beginning at 36 days, small vacuoles were seen scattered in the "chromophobic" anterior-lobe tissue but it was difficult to determine whether this vacuolation was intracellular or intercellular. However the 14-month implants had numerous large "chromophobes" which clearly contained large nonstaining vacuoles.

Anterior-Lobe Cytology

Two of the 3 staining methods used in this study stain differentially the 3 main classes of pituitary cells, traditionally called "basophils," "acidophils," and "chromophobes." "Basophils" are stained by the recolorized leucofuchsin of the PAS procedure and by aniline blue in the Mallory technique. Either of these stains will allow differentiation among "basophils" of "thyrotrophs" and "gonadotrophs" on a morphologic and distributional basis (*14*) in certain species, including the mouse. "Acidophils" are stained by the orange G counterstain of the PAS method, and by acid fuchsin in the Mallory method. "Chromophobes," as their name implies, are not stained by these nor by any of the traditional pituitary stains. However, some of the "chromophobes" are stained by basic dyes, *e.g.*, toluidine blue, by virtue of their ribonucleic

TABLE 1.—*Summary*

Days after implantation	No. of mice	Neural lobe	Intermediate lobe	Anterior lobe
				Condition of tissue
1	3	Present but variably intact or edematous (degenerate).	Generally not completely intact; variable amount of necrosis.	Much necrosis, blood-engorged sinusoids, edema, nuclear pyknosis.
3	3	"	"	"
5	3	"	"	"
8	3	Present but atrophied to small remnant.	"	"
11	3	"	Intact; small PAS-positive cells, some with negative Golgi image. Mitosis present. Volume of lobe decidedly increased.	Almost uniformly intact, predominantly vesicular nuclei, compact sinusoid arrangement.
15	3	No recognizable remnant in any case.	"	"
20	7	"	Cell size increased, staining somewhat increased, more frequent Golgi images.	"
25	2	"	"	"
36	3	"	Cell size definitely greater, Golgi images larger and more frequent, less mitosis, light and dark cells.	Some vacuolation of cytoplasm.
51	3	"	"	"
4½ mos.	2	"	"	"
14 mos.	7	"	"	Considerable numbers of vacuolated cells, some scattered pyknotic nuclei.

of histologic findings

Anterior lobe—Continued			
Acidophils	Basophils	Chromophobes	Cytoplasmic basophilia
Good numbers of acidophils in intact portions. Also many dead acidophils lacking stainable nuclei in interior necrotic areas.	Same comments as for acidophils.	Nothing unusual regarding chromophobes.	
"	"	"	
"	"	"	
"	"	"	
Good numbers present.	No definite basophils seen.	Some large 'phobes, no Golgi images.	Present but not obviously greater than in normal 11-day pituitary.
Variable numbers, probably less frequent than above.	A few pale PAS + cells, vesicular nuclei.	Some Golgi images in 'phobes: vesicular nuclei; nucleoli.	
"	"	"	Much greater than in normal 20-day-old pituitary. Heavy staining in cytoplasm *ca.* Golgi zone.
"	A few in one implant clear and pyknotic.	Increasing nos. of Golgi images.	Increasing cytoplasmic basophilia.
Markedly reduced numbers, with high proportion of pyknotic nuclei and shrunken cytoplasm.	Possibly a few in one implant.	(Less mitosis) Chromophobes begin to line up along sinusoids as "palisades" with nuclei in basal position.	"
"	None seen	"	"
"	"	Even more and larger Golgi, palisade formation throughout.	"
Hardly any seen.	"	Increased size of cells and nucleoli.	

TABLE 2.—*Implants in normal hosts and normal pituitaries*

Time after implantation (*days*)	Implants in normal hosts							
	Calculated volumes ($mm.^3$)				Percent of total volume			A/I
	Anterior lobe	Intermediate lobe	Neural lobe	Total	Ant.	Int.	Neu.	
15	0. 0449	0. 0209	—	0. 0658	68	32	—	2. 15
25	0. 1321	0. 0288	—	0. 1609	82	18	—	4. 58
36	0. 1476	0. 0454	—	0. 1930	76	24	—	3. 24
51	0. 2480	0. 1345	—	0. 3826	65	35	—	1. 84
Age and sex (*days*)	**Normal pituitaries**							
1 ?	0. 0382	0. 0049	0. 0058	0. 0480	78	10	12	7. 73
1 ?	0. 0390							
3 M	0. 0451	0. 0069	0. 0088	0. 0608	74	11	15	6. 51
3 F	0. 0749	0. 0135	0. 0127	0. 1013	74	13	13	5. 51
8 M	0. 0760	0. 0134	0. 0156	0. 1051	72	13	15	5. 64
26 F	0. 1612	0. 0404	0. 0249	0. 2266	71	18	11	3. 99
33 M	0. 3630	0. 0695	0. 0427	0. 4754	76	14	9	6. 88

acid content. Thus, in respect to basic dyes, these cells are not literally "chromophobes." For simplicity, the traditional terminology will be employed in this paper but the terms will be enclosed in quotation marks for reasons that will be indicated in the Discussion.

"Chromophils" were far less numerous in all the implants than in normal pituitaries. Among the implants the highest proportions of "chromophils" were seen in intact areas during the first week. After the first week, cells with specifically stainable granules became progressively more scarce. This loss of stainability was in contrast to the marked increase in chromophils which took place in the normal mouse pituitaries following the day of birth. Figure 22 illustrates a typical area from the lateral portion of a 54-day-old mouse pituitary. In contrast to the predominantly "chromophobic" anterior lobes of 51-day implants (fig. 23), both "basophils" and "acidophils" are numerous and large.

The nuclei of "acidophils" in 1- to 8-day implants were either vesicular or pyknotic, reflecting the state of relative intactness or degeneration of parts of the gland. In the 11- to 25-day implants well-granulated "acidophils" were present in all cases (fig. 21), though fewer than normal, and almost all had clear, vesicular nuclei with conspicuous nucleoli. In the 36-day, 51-day, and 4½-month implants, however, "acidophils" were extremely scarce and their nuclei were usually pyknotic. In these, the cytoplasm was generally reduced in size and often compressed in shape.

"Basophils," like "acidophils," could be recognized in some of the *intact* areas of anterior-lobe tissue of implants during the first week. Their nuclei were likewise either pyknotic or vesicular. Their cytoplasm stained faintly with aniline blue or with PAS, and was generally more voluminous than that of acidophils. In most of the remainder of the

series, cells having the tinctorial characteristics of "basophils" could not be differentiated even upon serial examination of sections. In 6 out of the 16 implants of 11 to 51 days, a *very few* cells could be discerned which stained *faintly* with aniline blue or PAS, and these were generally rather large cells as compared with "acidophils."

"Chromophobes" obviously constituted the major portion of anterior-lobe tissue of transplants older than 11 days. Beginning at 15 days, it was apparent that some of the "chromophobes," which were distinguished by their inability to take up the standard pituitary stains, differed morphologically from the classical "chromophobes" of normal pituitaries. Many of the nonstaining cells were conspicuously larger than is usual for "chromophobes," and, furthermore, negative Golgi images could clearly be seen in the cytoplasm. The proportion of total cells constituted by the large Golgi-containing "chromophobes" increased markedly with time, so that in the 51-day and 4½-month groups they were in large majority. These cells almost always had vesicular nuclei, and quite commonly had large, brilliantly stained nucleoli.

Two morphologically distinct types of "chromophobes" could be recognized, and these had a different distribution in the implanted anterior lobes. One type, small in number, was localized in those portions of anterior-lobe tissue which were adjacent to what would normally be the lateral and anterior portions of intermediate lobe and to the pars tuberalis (fig. 6). This area was characterized by Purves and Griesbach (*14*) as the "sex zone" where gonadotrophic "basophils" are concentrated. The cells in this area in the implants tended to be relatively large in comparison to the other anterior-lobe cells, and to have distinct, though nonstaining, granules in the cytoplasm. The presence and character of the cells in this area were rendered more conspicuous by their formation into acini demarcated by abundant connective tissue and sinusoids—a similar circumstance to that found in this region in the intact mouse pituitary gland. PAS-positive or aniline blue-positive cells, though infrequently seen, were most commonly found in this region.

The second type of "chromophobe," much more plentiful than the one described above, was distributed uniformly throughout the remainder of the anterior-lobe tissue (figs. 4, 5, and 6). The cytoplasm, unlike that of the first cell type described, was nongranular, and was smooth and homogeneous in appearance. Negative Golgi images were more readily distinguished in these cells. These more numerous "chromophobes" were arranged in a conspicuous "palisade" formation along the sinusoids (fig. 10). The "palisade" appearance was produced by the alignment of cells of uniform size and shape, with basilar nuclei and supranuclear Golgi images. Some evidence of palisade formation was first seen in the 20-day implants, but by 51 days these dominated the appearance of the anterior-lobe tissue.

Most of the "chromophobes" of the implants showed conspicuous cytoplasmic basophilia with toluidine blue, excepting those of the "sex zone" which were virtually unstained (fig. 7). This staining of the an-

terior-lobe cells and of a smaller proportion of intermediate-lobe cells was apparently due to ribonucleic acid, as all the cytoplasmic basophilia was destroyed by the ribonuclease pretreatment. The homogeneous mass of cytoplasmic nucleoprotein was characteristically localized peripherally in the cell producing a ring-shaped configuration enclosing the unstained Golgi area (fig. 9).

Pituitaries implanted for 11 and 20 days were compared as to degree of RNA staining with normal glands from 11- and 20-day-old mice. Figures 13 to 16 demonstrate the results and show that the normal glands from both age groups displayed very little staining, mostly of a diffuse character, but some perinuclear masses were present. In the 11-day implant there was an indication of the possible presence of more cytoplasmic basophilic material than in its normal counterpart, whereas, in the 20-day implant the amount of RNA had obviously increased and was localized in the typical fashion described above. Studies of later implants bore out the indication that the cytoplasmic RNA content may increase with time after implantation and that it occurs in larger concentration than in normal pituitaries of corresponding age.

In the normal intact pituitaries, RNA-containing cells (presumably "acidophils") were found primarily in the lateral areas of the gland while the "sex zone," and "basophils" generally, were negative. Within individual cells, RNA was most frequently seen as an amorphous mass of deeply staining material closely adherent to the nuclear wall, while occasionally it was present as a thin rim at the extreme periphery of the cell. Figures 11 and 12 illustrate the staining pattern for nucleoprotein compared with that of the PAS-positive material in the "sex zone" from a 2½-month-old female mouse.

Neural Lobe

The neural lobe could be recognized at 1, 3, and 5 days following implantation in either intact or degenerate form (figs. 1, 3, and 21). None of the subsequent implants contained any recognizable neural lobe.

Intermediate Lobe

In the first week after implantation, mitoses were rarely seen in the intermediate lobe. Numerous pyknotic nuclei were frequently seen and cells were generally very small and they stained poorly. However, by 11 days a definite increase in cell size, cytoplasmic staining, and frequency of mitosis was observed. In addition, negative Golgi images were clearly definable in the cytoplasm and the nuclei had generally become large and vesicular with prominent nucleoli. In the stages after 11 days, the cell size, the frequency and size of the Golgi images, and the size of nucleoli increased further, as did the apparent over-all size of the pars intermedia. At the same time, the staining intensity of the intermediate-lobe cells was only slightly increased. In the 36-day group and in all subsequent groups, some of the cells stained well with PAS or aniline blue, while others stained scarcely at all (fig. 18). A size difference in

the tinctorially distinct cells was striking: the nonstaining cells were larger and more frequently showed a large Golgi image.

The relatively greater increase in total volume of the intermediate lobe as compared with that of the anterior lobe is reflected in the ratios of calculated total anterior-lobe volume to intermediate-lobe volume for implants and for normal pituitaries (table 2).

Intermediate-lobe cells had far less histochemically demonstrable cytoplasmic basophilic material (RNA) (fig. 8). Many cells, in fact, did not stain at all, in contrast to the positive reaction of most anterior-lobe cells.

Pars Tuberalis

Cells of the pars tuberalis were seen in implants throughout the series (fig. 19). They were readily identified by their content of strongly PAS-positive, saliva-labile material, presumably glycogen (*15*). The glycogen in the epithelial layer of the cornea (*16*) surrounding the implants was also revealed by this method, and thereby provided additional control for the salivary digestion. Neither the corneal epithelium nor the pars tuberalis displayed metachromasia with toluidine-blue staining, while the mucopolysaccharide-containing substantia propria was strongly metachromatic. These results are consistent with the view that the material stained by PAS in the pars tuberalis is glycogen.

Fourteen Months after Implantation of Pituitaries

The 7 implants that had been allowed to remain in the eye 14 months were all very enlarged, and it was apparent from gross examination that they were considerably larger than normal adult mouse pituitaries. They consisted predominantly of anterior-lobe tissue in which no "chromophils" could be seen (fig. 24). The "chromophobes" of these implants were very enlarged, displayed greatly hypertrophied Golgi images, and were occasionally vacuolated. There was some evidence of palisade formation of cells along sinusoids as in the implants described above. Some nucleoli of tremendous size were seen which filled most of their respective nuclei.

In contrast to all the earlier implants, the intermediate lobe after 14 months comprised only a small fraction of the total implant. The cytology of the intermediate lobes, however, was similar to that in the several previous groups. In addition there were no longer any large empty "pockets" in the implants, these presumably having been filled up by growth of the tissue. Thus, between 4½ and 14 months, the intermediate lobe remained apparently unchanged, whereas the anterior lobe showed marked hyperactivity, hypertrophy, and hyperplasia.

Discussion

A large portion of some of the first-week implants was obviously necrotic tissue, and it is possible that functional effects of such "grafts"

might be due to absorption of hormones from dead cells rather than true secretion. It may also be reiterated that in the early implants the tissue was not uniform in condition throughout serial sections of the implant; the more intact tissues tended to be adjacent to the iris, the source of revascularization. These findings strongly indicate that, for a true assessment of the morphologic state of an implant, serial sections are required.

The degranulation of anterior-lobe cells after transplantation, observed in the present study, has been reported by previous observers (*2,3,5–7,10*). Degranulation has also been noted following stalk section (*17,18*) and hypothalamic lesions (*19*). Because of the impaired function of the anterior pituitary when severed from its connections with the hypothalamus, the scarcity of specific granulation has been interpreted as the cytologic manifestation of loss of activity (*20*). However, transplanted anterior lobe has been reported to be capable of maintaining a number of its usual functions, *i.e.*, stimulation of thyroidal iodine metabolism (*1*), release of corticotrophin in response to systemic stress (*6,7*), partial adrenal maintenance (*5*), and luteotrophin secretion (*21,22*). Since such implants are composed almost entirely of "chromophobes," it may be postulated that the "chromophobes" are, in fact, the elements responsible for some or all of the functional activity. Accordingly, at least some of the "chromophobes" may be poorly granulated "chromophils" whose rate of secretion exceeds that of the rate of synthesis. Supporting this concept are the findings in this study, first, of a rich cytoplasmic RNA content of the "chromophobes" of the implanted pituitaries, implying a high level of protein synthesis (*23*), and second, of the simultaneously occurring morphologic features (enlarged Golgi images and nucleoli and increased cell size) generally considered indicative of heightened secretory activity. Of course, as these cytochemical and cytologic features by themselves do not necessarily imply the elaboration and secretion of biologically active pituitary hormones, the synthetic activity of these cells could conceivably be, at least in part, of a nonhormonal nature.

That so-called "chromophobes" may be among the active secretory elements in pituitaries functioning *in situ* has been postulated previously on the basis of observations of estrogen-treated animals (*24*). In such pituitaries, enlarged cells showing cytologic evidence of activity were seen (enlarged Golgi and nucleoli and numerous mitochondria). Thus, as Severinghaus (*25*) has pointed out, before assigning a cell to the category of quiescent "stem-cell chromophobes," it seems necessary to observe other cytologic features in addition to its tinctorial properties.

The occurrence of increased cytoplasmic RNA in normal pituitaries has been observed following estrogen injections (*24,26*). Desclin (*26*) observed that the cytoplasmic basophilic masses were practically never seen in normal rat "basophils" or "chromophobes," and rarely in eosinophils, but they were abundant during pseudopregnancy, pregnancy, lactation, or estrone treatment in "chromophobes" and "acidophils." He also noted that while aniline blue stained the regular "basophils,"

both before and after ribonuclease treatment, this dye was poorly reactive on the cells that stained with basic dyes.[6] Wolfe (*24*) observed that during estrogen treatment, when both "acidophils" and "basophils" degranulate, the degranulating "acidophils" and the cytologically active "chromophobes" were characterized by intense cytoplasmic basophilia. Wolfe distinguished between "active" and "inactive" "chromophobes" on the basis of these cytologic criteria. Desclin and Gregoire (*2*) studied the morphology of pituitaries transplanted in the kidney for 15 days and found that estrogen injection resulted, as in their normal pituitaries, in greatly increased numbers of cells staining with basic dyes.[7] These cells were characterized by Golgi areas of larger dimension displacing the basophilic cytoplasm peripherally in the cell; thus they resembled the RNA-rich "chromophobes" of Wolfe's estrogen-stimulated pituitaries and those implanted in mice for 20 days and longer in the present study. Although the morphologic resemblance between the large "chromophobes" of the different preparations does not necessarily imply a functional similarity, the appearance of these cells in response to estrogen in both normal and implanted glands suggests that, in these circumstances, these cells are a morphologic expression of the functional response to estrogen. Furthermore, the findings that grafted pituitaries are capable of luteotrophin secretion with (*21*) and without (*22*) estrogen treatment are consistent with the idea that the large, RNA-rich "chromophobes," presumably degranulated "acidophils," are involved in luteotrophin production in the implants and in pituitaries *in situ* in the case of estrinized or lactating animals.

In view of the fact that the implanted rat pituitary was found capable of responding to stress (*6,7*), it is interesting to note that Finerty, Hess, and Binhammer (*28*) found increased cytoplasmic RNA in "acidophils" and "chromophobes" of normal rat pituitaries 1 hour and more after stress.

Thyrotrophin is known to be produced by transplanted mouse pituitaries (*1*). The PAS procedure is believed to stain thyrotrophin or its precursor in normal pituitaries, but since the anterior lobes of the implants were virtually negative to PAS after the first week, no information as to a possible localization of thyrotrophin production was forthcoming.

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[6] The traditional terminology for the pituitary cells has been criticized in the past as being inappropriate and unreasonable (*24*). Both "acidophils" and "basophils" are stained with *acid* dyes in the standard trichrome procedures; and "acidophils" have been stained with basic fuchsin. Further, "basophils" stain poorly, if at all, with any of the basic dyes, while both "acidophils" and "chromophobes" may exhibit a high degree of cytoplasmic basophilia. Because of the literal inaccuracy of these terms they have been enclosed in quotation marks throughout this paper. It should be recorded here that a recent publication (*27*) has presented evidence that throughout most of the *p*H range of the staining solutions, an acid dye, fast green, stained "acidophils" more intensely than "basophils," and a basic dye, methylene blue, stained "basophils" more intensely than "acidophils." The authors concluded on the basis of their study that the nomenclature acidophil, basophil, and chromophobe is informative and should be retained. However, the data were derived from the use of only 1 acid dye and 1 basic dye; at *p*H values between 6 and 7 the staining curves crossed so that the acid dye stained "basophils" more strongly than "acidophils" with increasing *p*H; and between *p*H 6 and 7 each dye stained either "acidophils" or "basophils" with equal intensity. Further, the authors point out that the acid dye aniline blue stains "basophils" more intensely than "acidophils" at each *p*H. Further work may show that unqualified retention of the old terminology is indeed reasonable. At present it might be preferable to remember that the names of the cells refer to distinct *morphologic* entities irrespective of tinctorial implications.

[7] Martins (*3*) also reported this effect in intra-ocular transplants using the methyl green-pyronine stain.

Because of the marked hyperplasia seen in the intermediate lobe following transplantation of the pituitary or following the induction of hypothalamic lesions, it was considered possible that the intermediate lobe might be the source of a thyrotrophic hormone which particularly stimulated the metabolism of radioiodine by the thyroid. Previous studies had indicated that thyroidal I^{131} metabolism was maintained to a much greater extent in both the above experimental circumstances than was thyroidal growth (*1*). Accordingly a series of mice was implanted with anterior lobe alone or with intermediate plus posterior lobe and a very small amount of anterior lobe. Functional studies indicated that all the thyrotrophic activity of transplanted pituitaries was accounted for by the anterior lobe alone (unpublished data).

Similarly, assays performed by Dr. R. W. Bates on isolated anterior, intermediate, or posterior lobe of both the rabbit and the rat showed that only anterior-lobe tissue caused thyroidal iodine depletion in the chick.

A preliminary experiment revealed no apparent morphologic differences between implants in animals which were: (a) untreated, (b) hypophysectomized, (c) hypophysectomized and castrated, (d) hypophysectomized and fed propylthiouracil.

The atrophy of the neural lobe in this series confirms the findings of many other investigations involving separation of the neural lobe from the brain. Atrophy of the neural lobe has been observed following transplantation (*5,8*), stalk section (*17,18*), and hypothalamic lesions (*29,30*). That it has not been reported more frequently in implants is probably due to the fact that in most studies only anterior lobe has been implanted.

The intermediate-lobe hypertrophy in the ocular implants of the present study may be compared to the findings of Etkin in the frog (*31*). Transplantation of the epithelial portions of the pituitary, either as a primordium or as the differentiated gland, resulted in hypertrophy and hyperplasia of the intermediate lobe, associated with excessive pigmentation of the host. Similar results were also produced by infundibular lesions. This suggested a restraining influence of the infundibulum on intermediate-lobe growth and activity. As far as we are aware, no previous reports have been made of intermediate-lobe hypertrophy in transplanted mammalian pituitaries. However, Brooks (*17*) found in the stalk-sectioned rabbit that, in comparison with anterior and posterior lobe, the intermediate lobe was much larger than normal and the cells were larger and stained more deeply. He described a granular formation in the cytoplasm, which may be the negative Golgi image and macula densa, and notes that these granular aggregates were never seen in normal glands. Barrnett and Greep (*18*) found that the intermediate lobe was usually enlarged after stalk section in the rat. Intermediate-lobe hypertrophy was reported in the rat, after hypothalamic lesions, by Bogdanove and Halmi (*30*) and has also been seen in this laboratory. Thus there are 4 types of preparations now reported in which interference with the brain-pituitary connections is followed by hypertrophy of the interme-

diate lobe. It may be of interest that intermediate-lobe hypertrophy is also seen in intact animals following certain hormone injections. Estrogen produced this effect very markedly in the hamster (*32,33*) and to a lesser extent in the rat (*34*). Corticotrophin caused hypertrophy and cytologic changes in intermediate-lobe cells in kittens (*35*).

Previous reports on the maintenance of pars tuberalis cells after transplantation have apparently not been made. It is possible that these cells are not generally observed due to their inconspicuous appearance when ordinary histologic methods are employed. The preservation of glycogen and its demonstration with PAS staining (*15*) made possible the identification of the pars tuberalis in these implanted pituitaries. Since the function of the pars tuberalis is not known, the significance of its persistence in implants cannot be surmised.

Summary

1) Pituitaries of newborn mice were implanted in the anterior eye chambers of normal adult female mice of the same derivative strain (BALB/c × C3H), and the resulting viable transplants taken for cytologic study at varying intervals from 1 day to 14 months.

2) It was found that during the first week following implantation (1–8 days), the pituitaries were in varying stages of degeneration or necrosis, with marked stasis of blood and engorgement of sinusoids. Large areas of dead tissue could frequently be seen in one interior cavity.

3) Anterior-lobe tissue grew well subsequent to the first week after implantation to the eye, though probably not so well as the intermediate lobe. The stainability of anterior-lobe cells was much less than that of the normal gland; "acidophils" appeared less frequently with time until they were very rare by the 51st day, while just a few "basophils" were discernible in a minority of the specimens in the 15- to 36-day groups.

4) The "chromophobes" of implanted anterior lobes were morphologically dissimilar to those present in normal pituitaries, being much larger and having cytologic manifestations of activity. At the later time intervals these "chromophobes" formed striking "palisade" patterns and comprised a larger proportion of the cell population.

5) The pars tuberalis with its content of glycogen survived well in the implants of this series. Mitoses were not observed in this lobe.

6) The intermediate lobe, after the regression of the first week, exhibited considerable capacity for growth, attaining a size comparable to that of normal *in situ* pituitaries of a corresponding age. Mitoses were numerous, and the cells stained well with PAS or aniline blue, while negative Golgi images were prominent in the ample cytoplasm.

7) The neural lobe could be seen in implants of 1 to 8 days; in some of these it had a relatively intact appearance, and at other times it was markedly degenerated. At 11 days, only atrophic remnants were identified, but by 15 days and in the remainder of the series the presence of the neural lobe or remnants thereof could not be ascertained.

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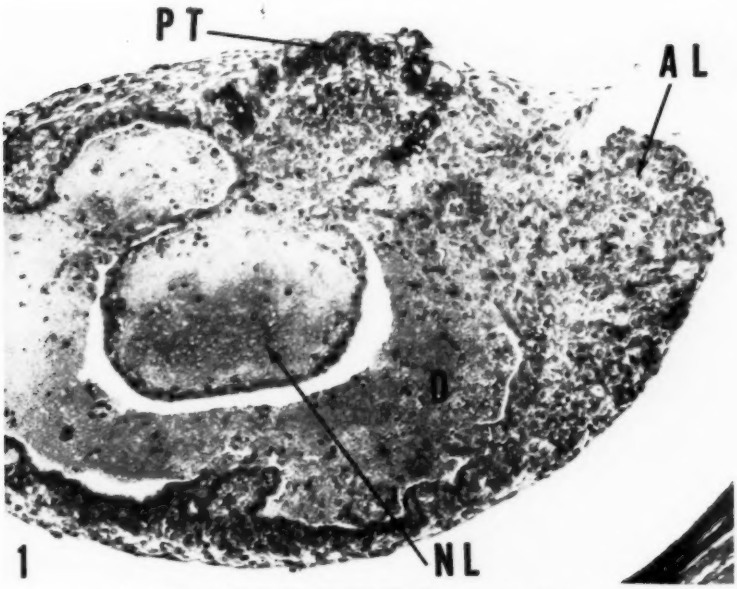
PLATE 37

FIGURE 1.—Pituitary taken from the anterior eye chamber 5 days after implantation, containing a large mass of debris, *D*, in the residual lumen. The neural lobe, *NL*, has lost most of its nuclei and stainable droplets; the intermediate lobe is only a thin layer. Pars tuberalis, *PT*, is strongly positive to PAS. Anterior lobe, *AL*, peripheral to the debris, is edematous and somewhat disorganized, and nuclei are vesicular. PAS and hematoxylin. × 150

FIGURE 2.—Higher magnification of a portion of the debris in the same implant as in figure 1, illustrating "dead" "chromophils" in which the nuclei no longer stain. Erythrocytes are also in the field. Modified Mallory. × 1250


PT
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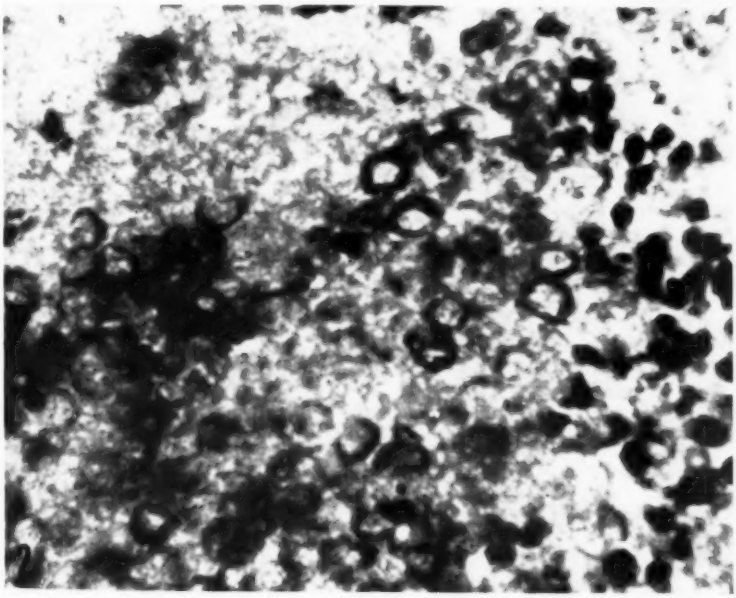
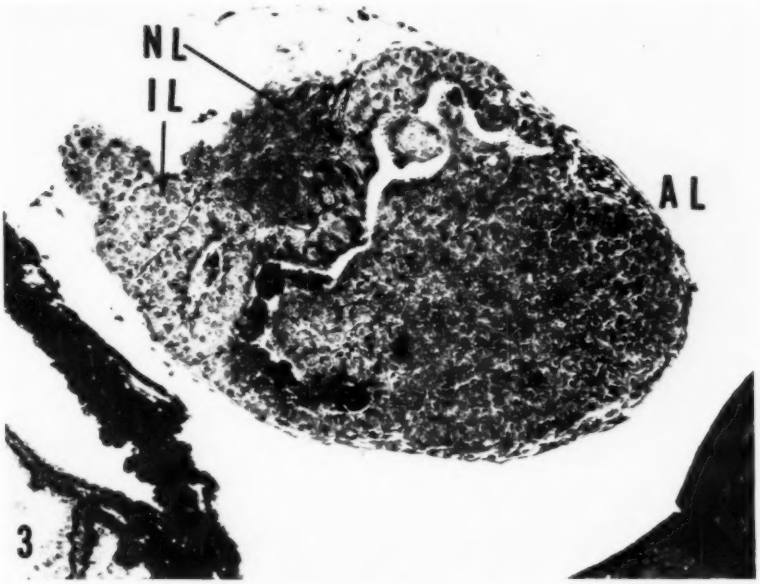


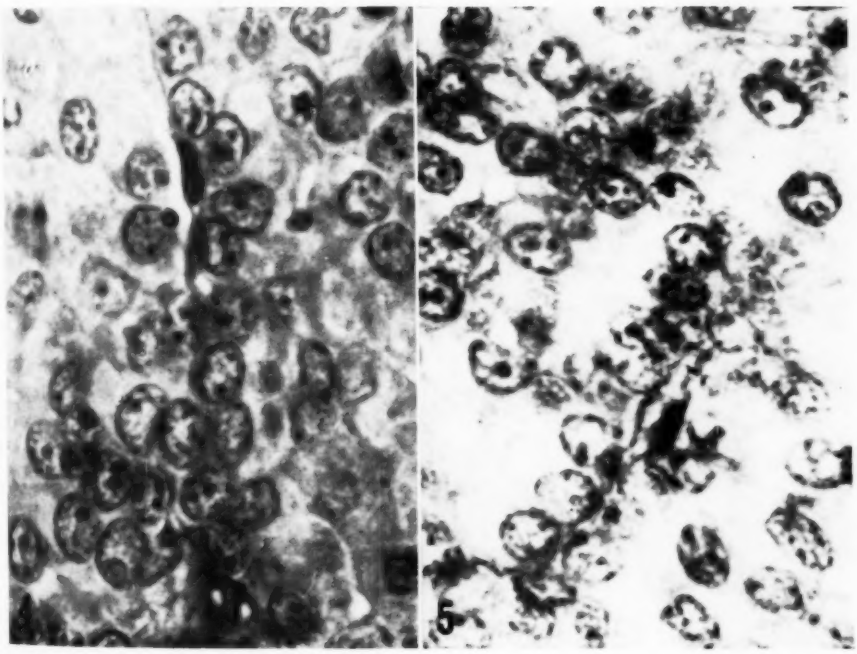
PLATE 38

FIGURE 3.—Another 5-day implant in which the 3 lobes appear intact. PAS and hematoxylin. × 150

FIGURE 4.—Anterior lobe of a 51-day implant illustrating "chromophobes" of large size, the nuclei of which are oriented toward sinusoid; large Golgi images, prominent nucleoli. Modified Mallory. × 1700

FIGURE 5.—Anterior lobe of another 51-day implant, similar features as in figure 4. PAS, hematoxylin, and orange G. × 1700





395335—56——9

PLATE 39

FIGURE 6.—Same 51-day implant as in figure 5. A blood vessel separates intermediate and anterior lobes. Just to the right of it is the area of anterior lobe containing many of the larger, more granular type of "chromophobes" surrounded by abundant connective tissue, and farther right are the predominating type of "chromophobes." PAS, hematoxylin, and orange G. × 360

FIGURE 7.—Another section from same implant as in figure 6. Demonstration of RNA in the cytoplasm. "Chromophobes" immediately to the right of the blood vessel, *BV*, did not take the stain, while those farther right stained well. Toluidine blue. × 360

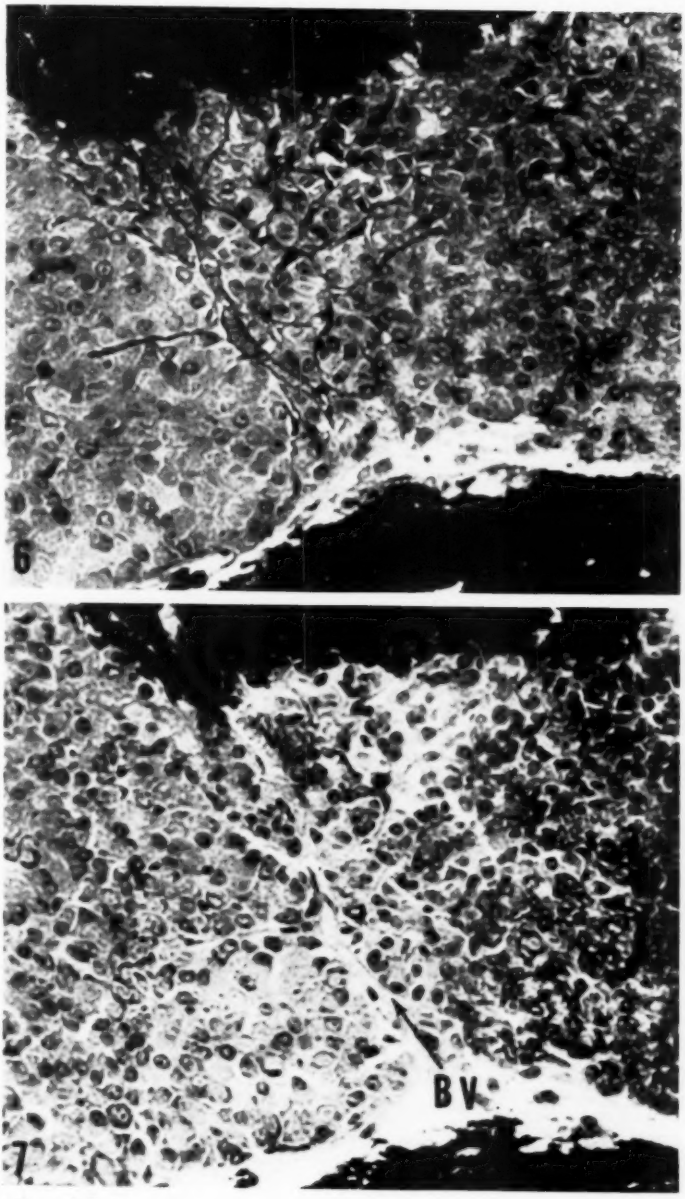
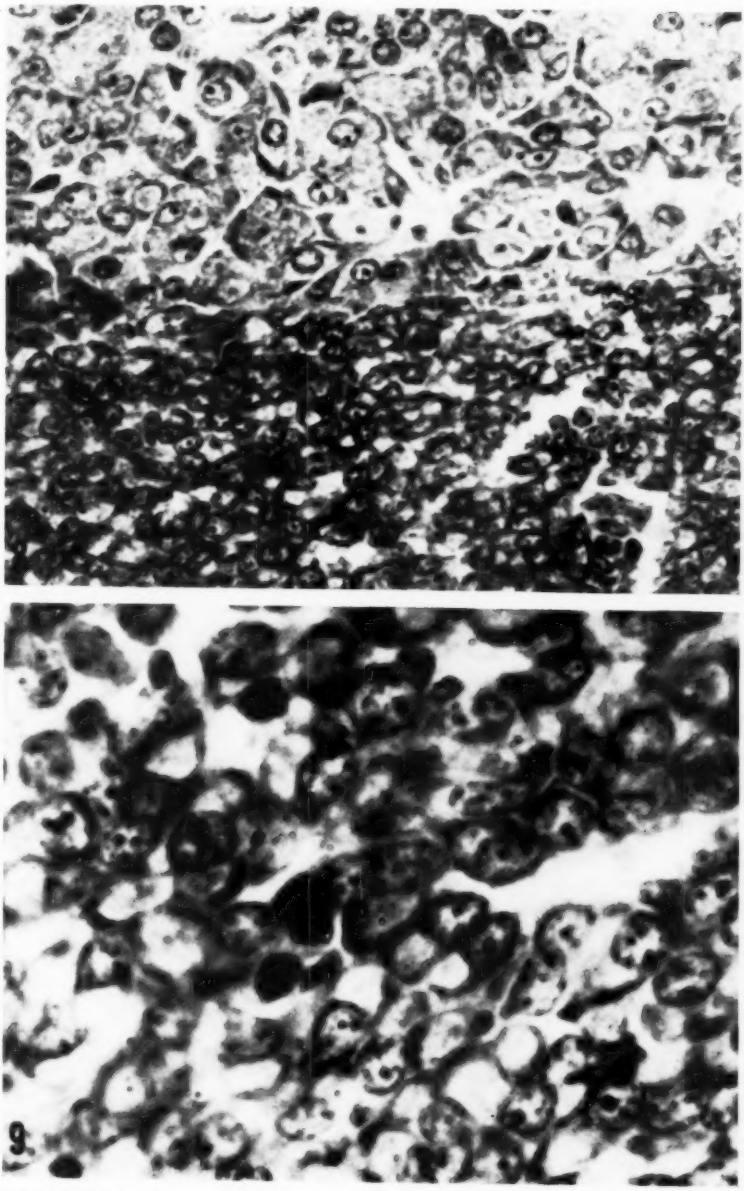

6
BV

PLATE 40

FIGURE 8.—Another area from same section as in figure 7: intermediate lobe in upper half of photograph, anterior lobe in lower half. Note differences in size of cells and pattern of RNA staining between *IL* cells and *AL* "chromophobes." Toluidine blue. × 690

FIGURE 9.—Higher magnification of field from same section illustrated in figures 7 and 8, showing typical RNA staining of cytoplasm and nonstaining Golgi zone of "chromophobes." × 1700



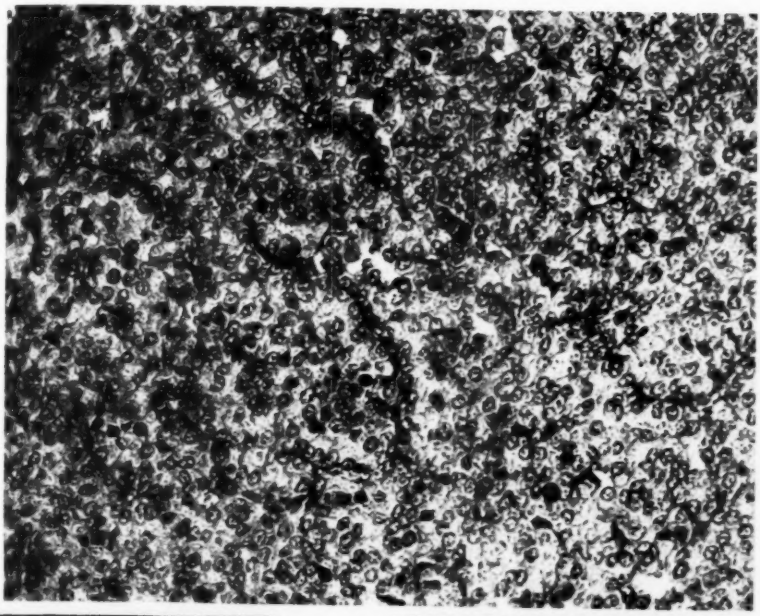
395335—56——10

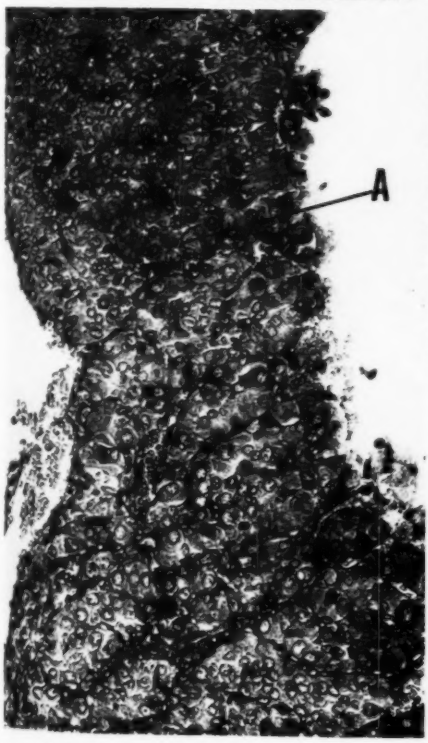
PLATE 41

FIGURE 10.—Anterior lobe of 51-day implant, "palisade" formation of nuclei of "chromophobes" along sinusoids, nonstaining cytoplasm with visible Golgi images oriented away from sinusoids. PAS, hematoxylin, and orange G. × 390

FIGURE 11.—*In situ* pituitary of female mouse 2½ months old (host to a 15-day implant). Most rostral and ventral area of anterior lobe, where greatest concentration of "basophils" is seen. "Basophils" appear gray in the photograph. The dark cells above the arrow are acidophils, A. PAS, hematoxylin, and orange G. × 280

FIGURE 12.—Another section from the same area of the pituitary shown in figure 11 for comparison of localization of PAS and RNA staining. The large "basophils" are very slightly stained or unstained, while considerable staining reaction is present in the "acidophil" area above. Note the intense perinuclear staining reaction in some of the cells. Toluidine blue. × 280

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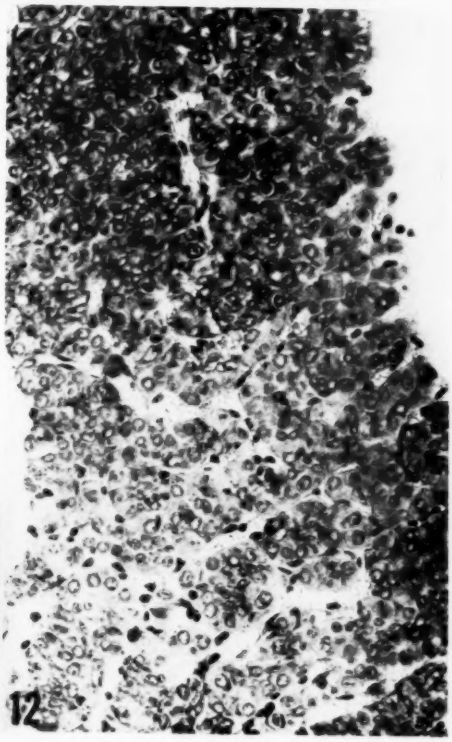

12

PLATE 42

All sections stained for RNA at the same time. In each case only nuclear staining was possible in adjacent sections pretreated with ribonuclease. Toluidine blue. × 690

FIGURE 13.—*In situ* anterior lobe of an 11-day-old normal male mouse. Only some diffuse staining of cytoplasm, except for a cell (*arrow*) in the approximate center with a dark perinuclear mass.

FIGURE 14.—Anterior lobe of 11-day implant, not obviously different from a normal implant.

FIGURE 15.—*In situ* anterior lobe of a normal 20-day-old female mouse.

FIGURE 16.—Twenty-day implant in which there are numerous large cells with peripheral masses of RNA typical of the older implants. In the center of the photograph a group of such cells is arranged in a circle bounded by connective tissue of a sinusoid.

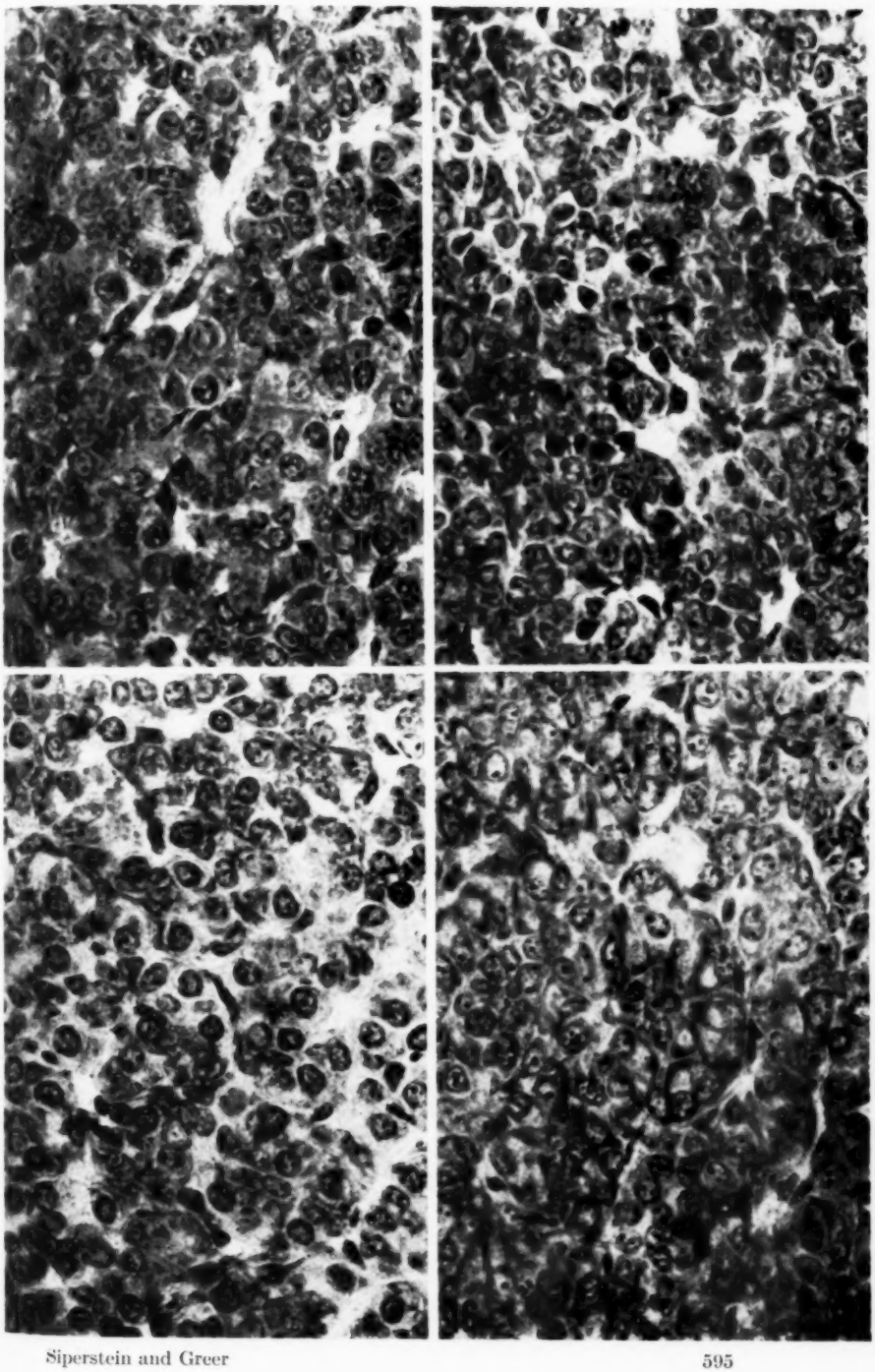


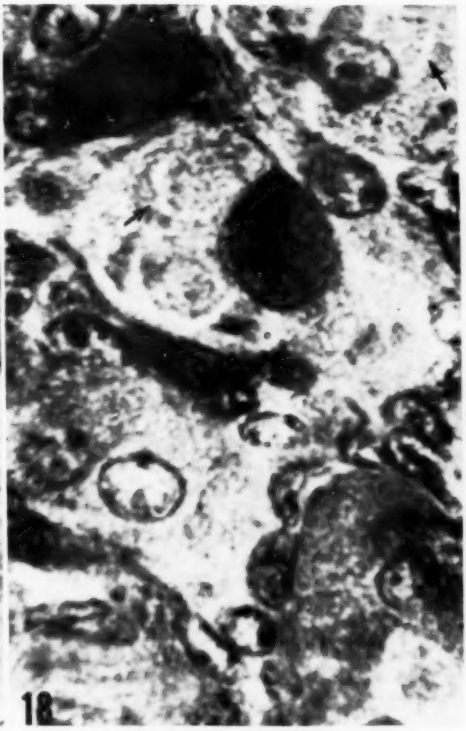
PLATE 43

FIGURE 17.—Intermediate lobe of a normal 54-day-old male mouse. Note homogeneous staining of cytoplasm, small Golgi (*arrows*). PAS, hematoxylin, and orange G. × 1700

FIGURE 18.—Intermediate lobe of a 51-day implant. Some cells much darker and smaller than most of the cells, very large Golgi images (*arrows*) in the large, paler cells. PAS, hematoxylin, and orange G. × 1700

FIGURE 19.—Fifteen-day implant with strongly PAS-positive pars tuberalis. Blood vessels passing through connective tissue from iris to implant. PAS and hematoxylin. × 210

FIGURE 20.—Section adjacent to that in figure 19, pars tuberalis is PAS-negative. (The nuclear staining is stronger in this section than in fig. 19.) Saliva, PAS, and hematoxylin. × 210



PT
TL

PT
TL

PLATE 44

FIGURE 21.—Eleven-day implant composed of healthy epithelial tissues. Large pouch containing some blood. "Chromophils" in piece of *AL* projecting into pouch. Mitoses in *IL*. PAS, hematoxylin, and orange G. × 176

FIGURE 22.—Anterior pituitary of a 54-day-old normal male mouse. "Acidophils" are yellow; PAS-positive cells, pink. Golgi images in cytoplasm of both types of "chromophils". PAS, hematoxylin, and orange G. × 1700

FIGURE 23.—Anterior lobe of a 51-day implant. Comparison with fig. 22 illustrates the difference in staining properties between the implant and a normal male mouse pituitary of comparable age. Note cytoplasmic detail, particularly of "palisade" cells for comparison with RNA staining (figs. 8 and 9). PAS, hematoxylin, and orange G. × 735

FIGURE 24.—Anterior lobe of an implant after 14 months in the eye. Note increased cell size, vacuoles, large nucleoli. Some extraordinarily large nucleoli not easily distinguished in the photograph, as they stain poorly. PAS, hematoxylin, and orange G. × 735

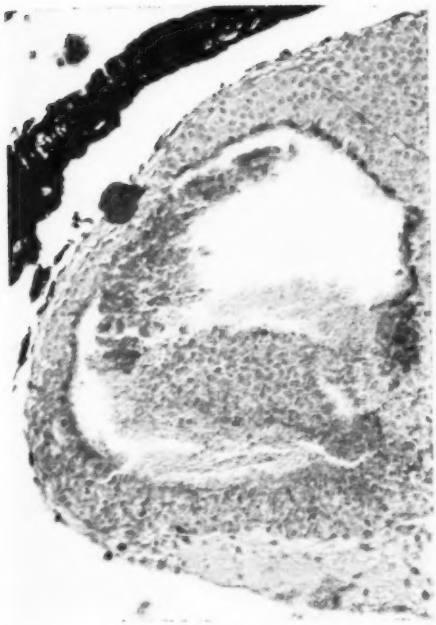


FIGURE 21.

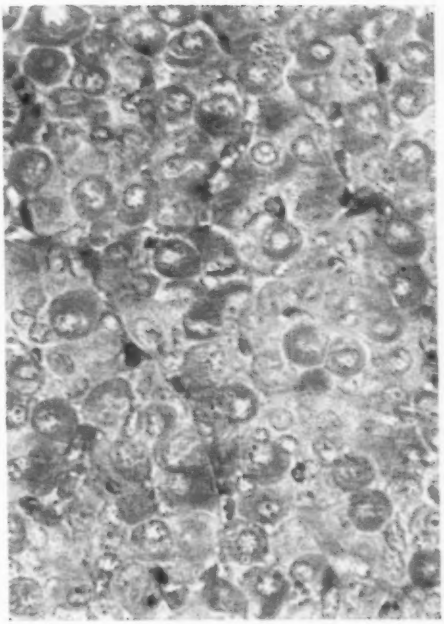


FIGURE 22.

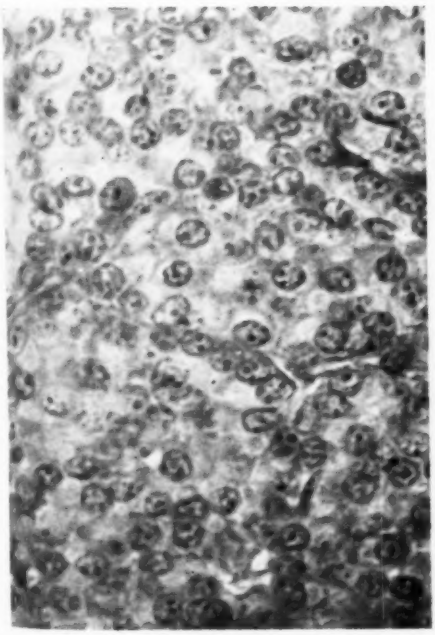


FIGURE 23.

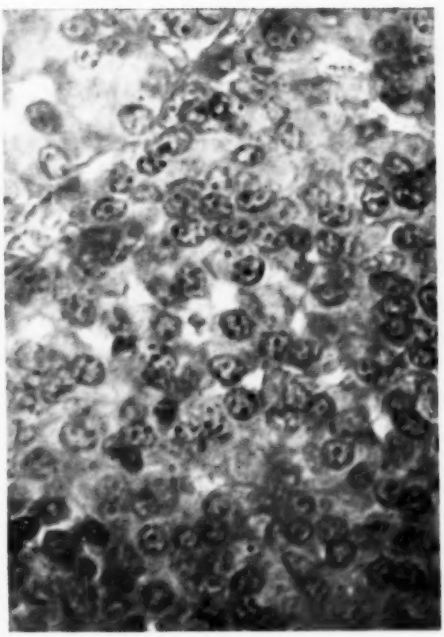


FIGURE 24.

Siperstein and Greer

Unusual Neoplastic Lesions of the Uterine Horns of Estrogen-Treated Mice [1,2]

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Tumors of the uterine cervix and upper vagina of estrogen-treated mice have been described (*1,2*). Tumors of the uterine cornua occurred rarely in mice. Lacassagne (*3*) described 1 very small carcinoma that was restricted to the endometrium of a uterine horn in which the central lumen was lined by a metaplastic squamous epithelium. This mouse had received prolonged treatment with estrogens and gonadotrophins. An undifferentiated uterine carcinoma was described by Woglom (*4*). This tumor resembled some reticulum-cell tumors (*5*). The uterus of the mouse is not infrequently the site of origin of, or involved in, reticulum-cell sarcomas.

One rather large uterine adenocarcinoma, noted recently in a mouse of 1 stock in this laboratory, and a number of smaller lesions, presumably of a similar type, are described below.

Materials and Methods

The experiments undertaken were designed to determine the effect of estrogens, administered either parenterally or topically, on cancer of the vagina and uterine cervix. Most of the mice used were from the BC stock,[3] although a few mice of several inbred strains were used. The significant aspect of this study is the description of the lesions rather than the genetic and quantitative data, since only the larger tumors or lesions were detectable, except accidentally, with the methods employed. All the mice in which lesions were observed were over 500 days of age at time of death, had been exposed to exogenous estrogen for 112 to over 300 days, and had had from 1 to at least 5 pregnancies prior to estrogen treatment (table 1). Two mice received weekly subcutaneous injections of 250 μg. of stilbestrol dissolved in 0.05 ml. of sesame oil; 5 received weekly

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[2] This investigation was supported in part by grants from the Anna Fuller Fund, the Jane Coffin Childs Memorial Fund, and by grant C-343 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[3] BC refers to a group of mice derived by hybridizing "PM" ♀ × C3H ♂ and backcrosses to the PM mice followed by brother × sister inbreeding. The PM mice have been mentioned in other papers (Cancer Res. 14: 204-209, 1954). Mice from the 25th to the 29th generations were used in this study. They were from several different sublines.

395335—56——10a

TABLE 1.—*Age and treatment of mice with uterine lesions*

Animal		Treatment* with estrogens	Age at start of treatment (*days*)	Period treated (*days*)	Age at death (*days*)	Other tumor or neoplastic lesions†
No.	Strain					
1	BC‡	16.6 μg .weekly	285	311	596	Stage IV—cervical cancer
2	BC	"	285	262	547	Stage II—cervical lesion
3	BC	"	426	147	573	
4	BC	"	365	255	620	Stage I
5	CBA	"	452	112	564	Mammary adenocarcinoma
6	BC	SS 250 μg. weekly	337	259	596	
7	BC	"	337	280	617	Stage I
8	BC	Intravaginal pellet	264	245	509	Stage IV

*Once each week 16.6 μg. of estradiol benzoate was injected in 0.05 cc. of sesame oil. Each week, 250 μg. of stilbestrol dissolved in 0.05 cc. of sesame oil was injected. Intravaginal pellet—a pellet weighing about 8 mg. and containing 1 part stilbestrol to 3 parts cholesterol was fixed in the upper vagina.

†Stages of uterocervical lesions were similar to those described earlier. Stage IV is considered to be invasive carcinoma (mouse #2).

‡Mouse #1 and mouse #2 each had a granulosa-cell tumor, which was transplanted subcutaneously when 53 days of age, but the transplant failed to grow. Mouse #3 also had a granulosa-cell tumor, transplanted subcutaneously, which failed to grow.

subcutaneous injections of 16.6 μg. of estradiol benzoate dissolved in 0.05 cc. of sesame oil; and 1 had a pellet of stilbestrol and cholesterol fixed in the upper vagina (1 part to 3 parts). The pellet was coated with colloidin and attached to a nylon thread which was passed through the cervix and attached to the uterus. In this way the pellet was held in place in the upper vagina.

Three of the mice had been recipients of grafts of 2 different granulosa-cell tumors. The grafted tumors were transplanted subcutaneously but since they had not grown after 220 days or more, the mice were transferred to other experiments. The transplants were not found at necropsy. The number of litters that these 3 mice had is unknown. The female and male mice were housed together in groups of not more than 6 to a cage, and the young were removed at birth. Other mice, which did not acquire the lesion to be described, had also received transplants of the same 2 ovarian tumors. These mice had been used in the breeding colony and the number of pregnancies and number of litters born and weaned were recorded and hence known.

Observations

One of the lesions was observed at necropsy in mouse #1 (table 1). When this mouse was 53 days old it received a subcutaneous graft of a granulosa-cell tumor. The tumor had not grown 233 days later. At necropsy, when the mouse was 596 days old, the grafted tissue could not be identified. The transplants had also failed to grow in 7 other recipients—the tumor was apparently not autonomous.

At necropsy a circumscribed, slightly pedunculated mass about 6 × 9 mm. in diameter was attached to the wall of the central part of an enlarged

and cystic left uterine horn (fig. 1). Superficially it resembled a reticulum-cell tumor, although such tumors usually appear nearer the cervix.

The uterine cervix was enlarged and the upper vaginal wall was slightly thickened. When examined under a dissecting microscope an epithelial lesion appeared to involve both the vaginal fornices and cervix.

Histologically, the uterine tumor was composed of moderately compactly arranged alveolar or tubular structures lined by a cuboidal epithelium (figs. 2 and 3). The surface of the tumor was covered by a similar epithelium and was apparently continuous with some of the deeper tubular structures. The epithelial layer replaced the normal serosal mesothelium. The lesion, insofar as the continuity of the tubules or clefts with the free surface of the tumor was concerned, resembled, superficially, the tubular adenomas found in ovaries under appropriate conditions. The stromal collagen formed an open network and was moderately vascularized. Mitotic figures were moderately numerous. The uterine glands did not contact the tumor at any point, although the myometrium was invaded at the place of attachment.

At first it was thought that the tumor might be an endometrial adenocarcinoma. No other lesion of this type in mice had been noted previously. The possibility of its being a metastasis of the uterocervical cancer was slight.

The uterocervical lesion was an invasive epidermoid carcinoma (Stage IV; figs. 4 and 5). The entire cervix was replaced by tumor and some extension occurred into 1 vaginal fornix. Dorsally, the cancer had spread to the rectum but in no place did it transgress the serosa. The entire area was sectioned and 2 out of every 10 sections cut at 8μ were examined.

The right uterine horn was enlarged; the serosa was intact and the myometrium well developed. The endometrial stroma was almost entirely hyaline. Plasma cells and polymorphonuclear leukocytes were accumulated in small numbers near the luminal basement membrane. Most of the glands were cystic and distended.

A second lesion appeared in a mouse (#2, table 1) treated in the same way as mouse #1, except that it was killed when 547 days old. No uterine lesions were noted at necropsy. The uterine cervix was enlarged but a uterocervical tumor was not noted. The right uterine horn contained an abscess.

A small nodule of alveolar tissue, not over 0.5 mm. in diameter and covered by a columnar epithelium, was attached to the uterus about halfway between the mesometrial and antimesometrial surfaces. Except for size the growth was similar to the larger one previously described. The uterine horn in the area involved showed no pyometra; the endometrium was largely hyaline. Only 7 sections of each uterine horn were available. The growth was limited to 1 uterine horn.

An epithelial lesion (Stage II) involved the lower half of the uterine cervix. The lesion was composed of well-differentiated stratified squamous epithelium.

Another mouse (#3, table 1) had 1 nodule that measured about 0.5 mm. in diameter and was composed of small alveoli. Although several mm. segments of both uterine horns were sectioned only 1 nodule was found. It was attached at the junction of the uterus and mesometrium. The surface of the lesion was covered by mesothelium. Mitotic figures were quite numerous. Pyometra was lacking. The uterine cervix and vagina showed no epithelial lesions.

Several mm. of both uterine horns of another mouse (#4, table 1) were sectioned serially. One nodule was located on the antimesometrial border of one uterine horn at one level (figs. 6 and 7). A few mm. away another growth was located at the uteromesometrial junction. This lesion, unlike the other, was composed of multiple papillary projections from the serosal surface. Only a few alveolar nodules were found in the subserosal or outer muscular layers. Mitotic figures were abundant in both nodules. Two small nodules were found at or near the uteromesometrial junction of the other uterine horn. The uterine cervix had a small area of epithelial ingrowth rated as Stage I. This small lesion was located near the ventral aspect of the cervix near the external os.

Two lesions were detected, 1 on the lateral side of one uterine horn and 1 on the distal mesometrium of a strain CBA mouse (#5, table 1). One lesion was less than 1 mm. in diameter and was composed of small, compact alveoli (fig. 9). The other was over 2 mm. long and of the papillary type. In some areas the lesion was only 1 cell thick, resembling a thickening of the serosa. In a few regions the cells extended into the serosal areas, apparently into lymphatics. The uterine horns were very large, the well-developed glands were largely cystic and hyperplastic. The uterine cervix was without epithelial lesions.

Two mice (#6 and #7, table 1) were sisters, and both, after serving as breeding animals for the first 337 days, were given weekly injections of 250 μg. of stilbestrol for the remainder of their lives. Both mice had small lesions about 1 mm. in diameter at the uteromesometrial junction. One nodule was located predominantly in the outer myometrium; the second was papillary (figs. 8, 10, and 11). One of the mice had a very small and somewhat questionable uterocervical lesion.

A very small uterine subserosal nodule was found in the last mouse (#8, table 1). This nodule, about 0.5 mm. in diameter, was located just distal to the uteromesometrial junction. The mouse had a large epidermoid carcinoma of the upper vagina and cervix in relation to the intravaginal pellet. Lateral to the vaginal orifice was a tumor measuring about 15 mm. in diameter. Its origin, however, was not related to the genital tract.

Discussion

The small uterine subserosal lesions are thought to be similar to the 1 larger tumor, except that they are at earlier stages. The tumors were multiple, as many as 3 separate tumors being found on the few mm. segments of one uterine horn in one instance. The greater number of lesions

appeared at, or near, the uteromesometrial junction. Quantitative aspects of the study are unsatisfactory, however, because the smaller lesions were not noted until after the uterine horns were sectioned for microscopic examination. Only sections 4 to 6 mm. in length from the central part of each uterine horn were preserved for histologic study. Similar lesions may have occurred at other areas. Also, similar lesions may have been present in uteri of other animals and not detected. Under the circumstances of the experiment an incidence of approximately 5 percent would be a rough estimate. The fact that in 2 instances 2 sisters had lesions indicates a possible familial influence.

The possibility of serosal metastasis from tumors of cervical origin was considered but excluded because only 2 mice had extensive cervical lesions. Although pyometra is not rare in mice given estrogens, and most mice given estrogens have septic uteri at some time, the lesions could not be associated with uterine or peritoneal infections (*6,7*). No adhesions were noted in any of the animals. The lesions could not be considered to be granulomatous.

Endometriosis has not been described as occurring in mice insofar as is known. The lesions might have had such an origin. The multiparity of all the mice was a common feature, and in this respect the mice differed from those studied earlier (*2,6,8*). In no instance, however, were cells observed that resembled uteroluminal epithelium. The resemblance to the deeper parts of the endometrial glands was not too striking. The possible development of the tumors from embryologic müllerian remnants seems plausible. The cells resembled somewhat those of ovarian germinal epithelium of the ovary. The larger lesions—1 mm. or more in diameter—were, however, quite unlike the larger ovarian tubular adenomas.

Furthermore, none of the ovaries among the mice of this group showed germinal epithelial ingrowths. All ovaries were similar to those of animals of comparable age receiving similar treatment.

The tumors seemed to arise from the serosa or from the serosa and underlying lymphatic capillaries. The subserosal area of the uterus of estrogen-treated mice contains abundant lymphatics. The infiltrating epithelial growths encroach upon the lymphatics rather quickly.

The uterocervical tumors were of the type observed among many estrogen-treated mice (*2,6,8*). The "nontumorous" areas of the uterus were also characteristic of the treatment. Most animals showed at least some cystic glands and most of the uteri had a pink-staining hyaline endometrial stroma (*6*).

Summary

1) Eight multiparous mice from 2 inbred strains that had been exposed to estrogens, either intravaginally applied or injected subcutaneously, had subserosal uterine lesions, adenomas, or adenocarcinomas.

2) Five of the mice also had infiltrative uterocervical lesions, 2 of which were considered to be epidermoid carcinomas.

395835—56——11

3) The subserosal lesions were considered to arise either in proliferation of müllerian rests persisting along the serosa, from the serosa, or from endometriosis.

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PLATE 45

All sections were cut at 7 or 10μ and stained with hematoxylin and triosin

FIGURE 1.—Photomicrograph of a cross section of the tumor on the uterine horn of a mouse (#1, table 1). A portion of the wall of the cystic uterine horn is located on the *left* side of the photograph. The frames indicate the areas shown in figures 2 and 3. × 14

FIGURE 2.—Photomicrograph of the area at the junction of tumor and uterus. A portion of a uterine gland is at the *mid-left* border. The myometrium was invaded by the tumor. The tumor at this region is continuous with the serosa. × 90

FIGURE 3.—Photomicrograph of a peripheral area of the uterine tumor. × 270

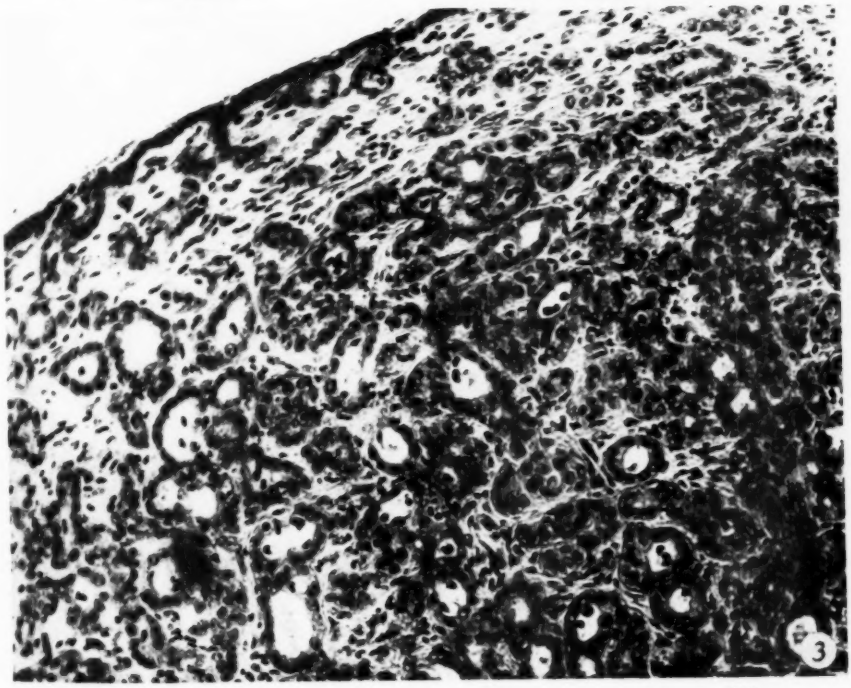

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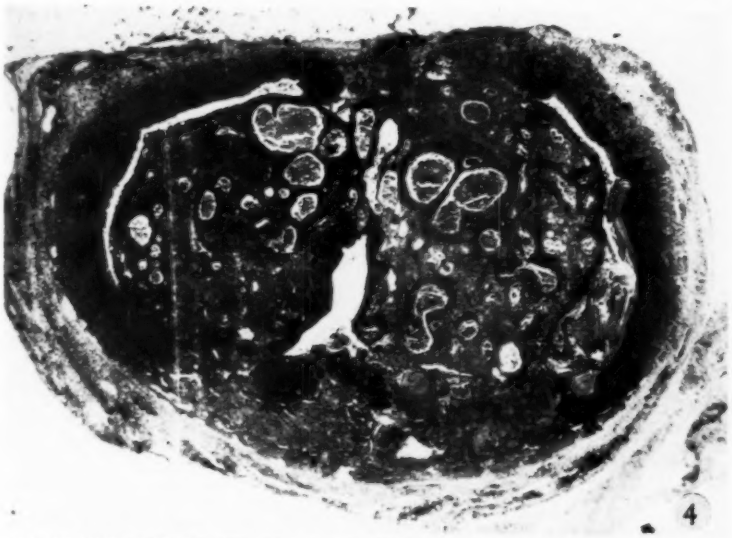
PLATE 46

FIGURE 4.—Cross section through the cervix and vaginal fornices of a mouse (#1, table 1). The entire cervix is invaded by epidermoid carcinoma (Stage IV). × 20

FIGURE 5.—Photomicrograph of an area of the above tumor. × 90



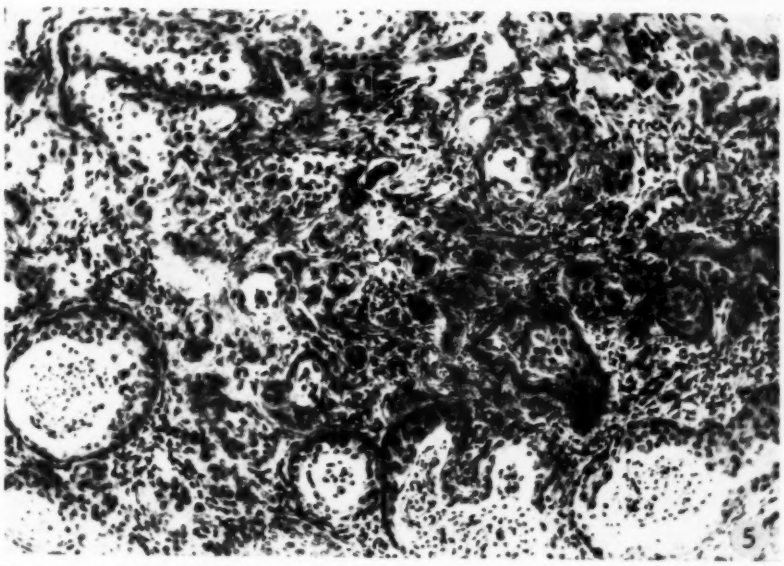


PLATE 47

FIGURE 6.—Cross section of a uterine horn of a 620-day-old mouse (#4, table 1). The small nodule is subserosally located at the antimesometrial border. Another small lesion appeared at the uteromesometrial junction at another level. × 18

FIGURE 7.—Photomicrograph of lesion shown in figure 6. × 90

FIGURE 8.—Photomicrograph of 1 of the 2 uterine lesions in a mouse 617 days old (#7, table 1). This lesion appeared at the antimesometrial border at a different level of the uterine horn than illustrated in figure 10. × 90

FIGURE 9.—Photomicrograph of a small uterine tumor of a 564-day-old mouse (#5, table 1). × 90

6

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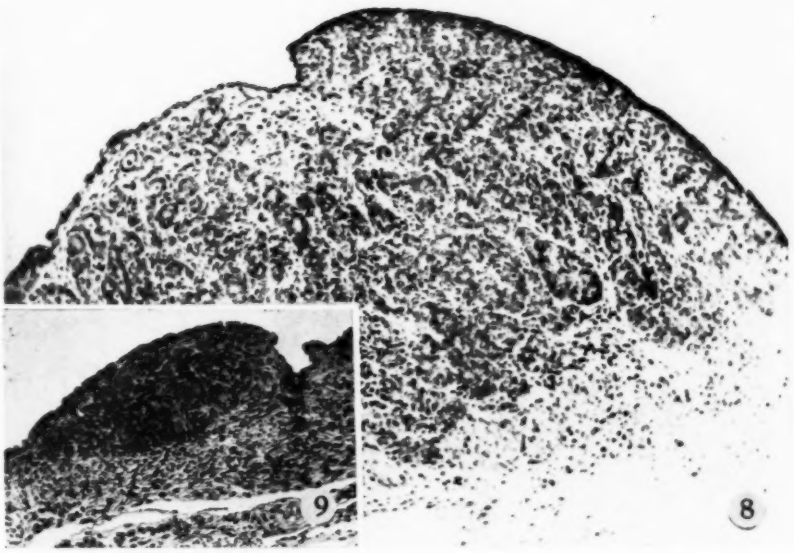
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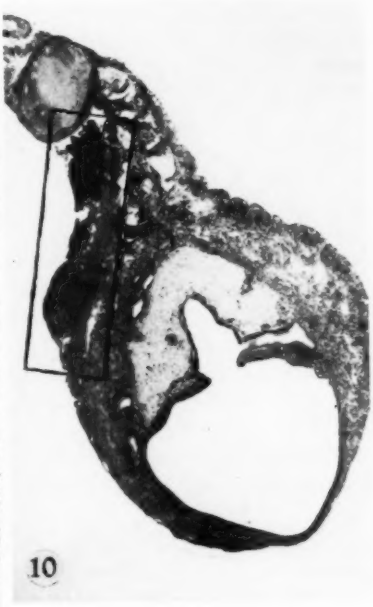
PLATE 48

FIGURE 10.—Photograph of a cross section of the uterine horn of a mouse 596 days old (#6, table 1) showing the cystic lumen, hyaline stroma, and lesion at the utero mesometrial junction. *Inset* shows area illustrated in figure 11. × 18

FIGURE 11.—*See* figure 10 above. × 90



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11

The Influence of Succinate and 2,4-Dinitrophenol on the Respiratory Activity of Mouse Cancers and Normal Tissues [1]

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That tumor metabolism is characterized by both an abnormally high glycolytic capacity and a relatively low respiration was first recognized by Warburg in 1923 (*1*). Recently (*2*, p. 310) he has reaffirmed and elaborated the view that ". . . for cancer formation there is necessary not only an irreversible damaging of the respiration but also an increase in the fermentation—indeed, such an increase of the fermentation that the failure of respiration is compensated for energetically . . . ." Among examples of numerous externally applied agents which may damage respiration in an irreversible manner Warburg cities (*a*) removal of oxygen, (*b*) use of respiratory poisons, and (*c*) treatment with X rays. He states that ". . . any respiratory injury due to lack of energy, however, whether it is produced by oxygen deficiency or by respiratory poisons, must be cumulative since it is irreversible . . . . ." (*2*, p. 310). Under such conditions, as Warburg points out (*2*, p. 309), ". . . both structure and respiration disappear . . . ." to a variable extent, and he suggests (*2*, p. 310) that " . . . the respiration connected with the grana remains damaged when it has once been damaged . . . . " This concept implies that an imposed energy deficiency results in permanent damage to the structure and respiratory function of the mitochondria, and that this damaged state is inherited if cancer is to ensue.

In the present study, measurements were made of the *in vitro* respiratory capacities of slices or whole cells of S91 melanoma, Krebs-2 ascites carcinoma, adult normal tissues, and growing normal tissues in the presence or absence of succinate, and/or 2,4-dinitrophenol. In addition normal brain tissues were subjected to anaerobic conditions, and alterations in subsequently measured metabolic activities in the presence of oxygen were noted. The data do not support the view that the relatively low Q_{O_2} values of the tumors studied are the result of an inherited irreversible damage of the type just referred to (*2*), since oxygen consumption could be elevated by removal of respiratory restraint (2,4-dinitrophenol) to approximately the range observed in normal tissues compared under similar conditions.

[1] Received for publication June 27, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Furthermore, normal brain, damaged by previous exposure to lack of oxygen, displayed a pattern of metabolic abnormality very different from that of the tumors. However, the data indicate that the tumors do fall within the range of tissue modifications previously characterized by Warburg as resulting from an " . . . acceleration of glycolysis without corresponding acceleration of respiration, or obstruction of respiration without corresponding impedence of glycolysis . . . " (*1*, p. 148). The data further show that a low response to exogenous succinate is characteristic of certain nonneoplastic growing tissues as well as of tumors. Factors affecting the magnitude of the *in vitro* succinate response are considered.

Materials and Methods

The S91 melanoma was maintained by intramuscular transplantation in C × DBA F_1 hybrid mice or in DBA mice. The Krebs-2 carcinoma was grown in the ascites form by intraperitoneal injection in the BALB/c strain of mice or in C × DBA F_1 mice. No differences attributable to host strain were encountered. Methods of preparing tissue for study, media used for *in vitro* incubation of tissue slices, etc., were as reported previously (*3*,*4*) with the following exceptions. Since, in measuring the response of tissue slices to succinate, it is important to remove as far as possible the remains of broken or crushed cells, tissues after sectioning were washed once or twice by suspension in a large volume of the appropriate medium, and centrifuged at 500 to 1000 r.p.m. in a Servall SS-1 angle-head centrifuge or, in many cases, removed by simply decanting the supernatant fluid. The sedimented tissue was then drained on filter paper before rapid weighing on a torsion balance. In the case of ascites cells, freshly extracted ascitic fluid (plus heparin) was spun for about 5 minutes at 500 to 1000 r.p.m. The sedimented pellet of tumor cells was drained on filter paper before weighing. Such cells had a dry weight of approximately 16 percent, and this value was used in calculating all Krebs-2 quotients. For metabolic determinations, weighed aliquots of the sedimented ascites cells were transferred to Warburg vessels, which had been previously filled with the appropriate medium.

Ascitic fluid, or normal mouse serum, before being used as a suspension medium for tissue slices, or for runs involving the ascites cells, was spun for 5 minutes at 19,000 r.p.m. The ascitic serum or normal serum, so obtained, was usually adjusted with *ca.* 0.1 *N* HCl to *p*H 6.8 to 7.2. Additional $KHCO_3$ or $NaHCO_3$ (0.0025 to 0.01 *M*) was added according to the anticipated rate of acid formation. The respiration of tissue slices was always measured with 100 percent oxygen in the gas phase, whereas Krebs-2 ascites cells were measured in air. CO_2 was absorbed with 4 *N* NaOH plus a filter-paper wick in the center well of the Warburg vessel. Q_{O_2} values represent the μl. of oxygen consumed per mg. dry weight per hour. While the initial *p*H in most cases was in the range 6.8 to 7.6, it sometimes became elevated during a run because of carbonate formation. However, control experiments at lower *p*H values demonstrated that this factor did not materially alter the results obtained. Comparisons of Q_{O_2}

obtained at constant CO_2 pressure (Summerson, differential manometry) with those observed in the presence of bicarbonate and NaOH showed no important differences.

Polyvinylpyrrolidone (PVP) was obtained from the General Aniline and Film Corporation under the trade name "Plasdone C" (control no. 3-GA-PVP-251-1). PVP solutions were adjusted to *p*H 6.8 to 7.2 with NaOH, and bicarbonate was added in the range 0.005 to 0.01 *M* as indicated by the anticipated rates of acid formation. The coenzyme I preparations were obtained from Nutritional Biochemicals Corporation, and had a rated purity of 95 percent. Sodium succinate, Merck ($Na_2C_4H_4O_4 \cdot 6H_2O$) was used in all experiments at 0.03 *M* concentration unless stated otherwise.

The following abbreviations are used throughout this paper: KR for Krebs-Ringer solution, MS for heparinized normal mouse serum, MAS for centrifugally cleared, heparinized Krebs-2 ascites-tumor extract, and 2,4-DNP for 2,4-dinitrophenol. Data of representative experiments are presented in the tables and text-figures.

Experiments and Observations

Steady-rate Q_{O_2}.—The metabolic quotients of Krebs-2 ascites cells were in the range reported by Warburg for the Ehrlich ascites cancer (*2*, p. 312). The steady-rate Q_{O_2} in KR with 0.2 percent glucose was in the range 5.0 to 7.0, and 7.0 to 10.0 without glucose. In MAS the values were 15 to 30 percent higher. Table 1 compares representative steady-rate Q_{O_2} values of Krebs-2 ascites cells and slices of S91 melanoma with those of several normal-growing tissues (chorion, embryo head), and nongrowing normal tissues (kidney, liver, brain). As in the case of Krebs-2 ascites tumor, the Q_{O_2} values of all tissues were generally higher in MAS than in KR. With the exception of embryonic head tissue, the steady-rate Q_{O_2} values in MAS of the tumors were lower than in normal tissues. As emphasized many years ago by Warburg (*1*), the importance of employing a medium-like serum in comparing Q_{O_2} values of normal and malignant tissues is obvious from table 1.

Effect of succinate.—As reported by many investigators [reviewed by Greenstein (*5*)] the percentage response of the tumors (table 1) to succinate was lower than in nongrowing normal tissues. However, in certain growing normal tissues (chorion, embryo head) the response to succinate was of the same order as that characteristic for tumors. These results make it clear that a low response to exogenous succinate is not a property limited to malignant cells, or necessarily to normal cells having a high basal Q_{O_2} (above 15.0). Thus embryonic head tissue, with Q_{O_2} values between 8.1 and 9.7 in KR, showed succinate responses of only 21 to 26 percent.

The following factors tended to *increase* the percentage response of the Q_{O_2} to added succinate: (*a*) excessive mechanical injury due to sectioning, (*b*) failure to wash out the debris of broken cells after sectioning, and (*c*) use of a purely saline medium.

The following factors tended to *diminish* the percentage response of Q_{O_2} to added succinate (*cf.* tables 1, 2, and 3): (*a*) the addition of PVP or PVP plus sucrose to the KR or substitution of MAS, MS, or serum-containing medium (*6*), and (*b*) the addition of coenzyme I.

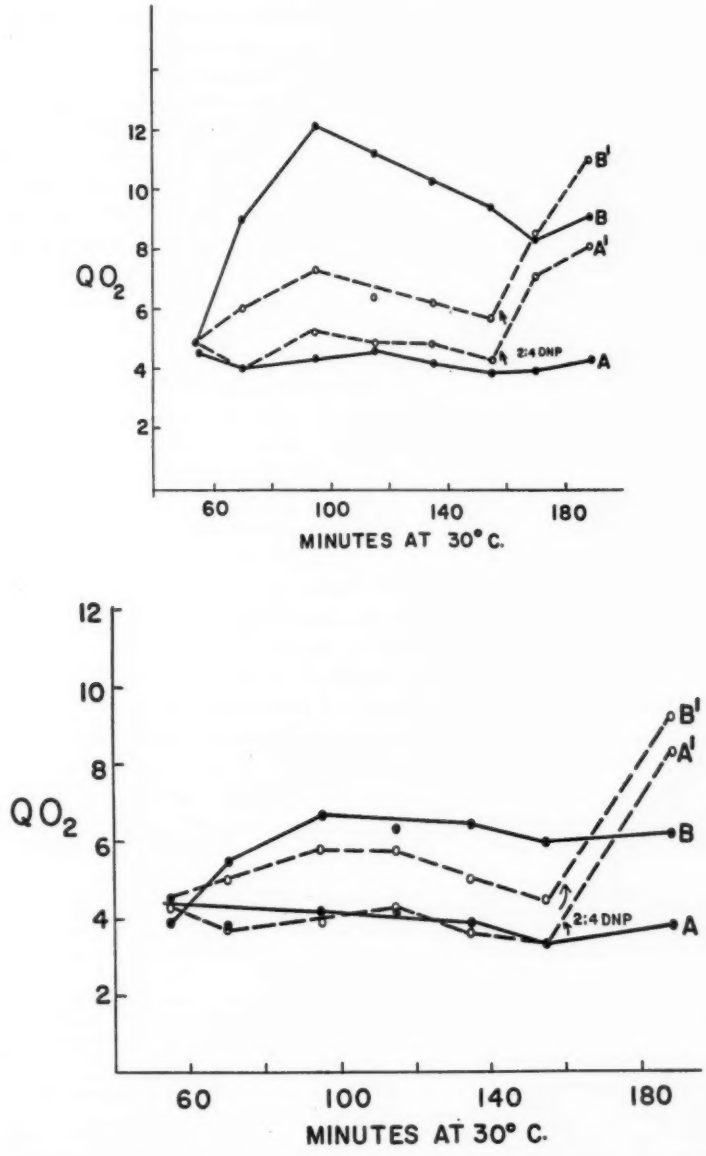
The Q_{O_2} time curves for S91 melanoma slices at 30° C. in KR with 0.2 percent glucose are shown in text-figure 1 (*top*). While the rate with

TABLE 1.—*Respiratory activities of normal and malignant mouse tissues at 38° C. with 0.2 percent glucose plus or minus 0.03 M succinate* *

Tissue and medium	Q_{O_2} at 38° C.		Succinate effect (*percent*)
	−succinate	+succinate	
Kidney			
MAS†	21. 1	26. 9	27
KR†	16. 1	33. 3	107
Liver			
MAS†	12. 0	19. 7	64
KR†	5. 7	27. 5	382
Brain			
MAS†	14. 1	20. 1	43
KR†	8. 8	16. 8	91
Unsectioned chorion (14-day embryos)			
MAS	21. 3	21. 9	3
KR	13. 7	15. 4	12
Embryo head (12 days)			
MAS†	10. 2	10. 8	6
KR†	8. 1	10. 2	26
Embryo head (14 days)			
Amniotic fluid	11. 1	13. 9	25
KR	9. 7	11. 7	21
S91 melanoma			
MAS†	9. 8	11. 7	19
KR†	7. 0	10. 5	50
MAS†	10. 0	10. 0	0
KR†	8. 4	12. 1	44
Krebs-2 ascites tumor			
MAS†	8. 6	9. 4	9
KR†	7. 5	8. 9	19
MAS†	8. 6	9. 9	15
KR‡	7. 4	9. 6	30

*Brackets designate data of individual experiments involving aliquots of the same tissue.
†Sectioned and washed in MAS before weighing.
‡Washed twice in KR before weighings.

added coenzyme I was slightly higher than that of the control, a marked diminution in succinate rate resulted from the addition of coenzyme. Addition of 2,4-DNP rapidly removed the restraint of Q_{O_2} caused by coenzyme. The addition of 10 percent PVP together with 5 percent sucrose (text-fig. 1, *bottom*) lowered the Q_{O_2} with succinate to almost the same extent as did coenzyme. Addition of 2,4-DNP rapidly removed the respiratory restraint occasioned by the combined action of PVP-



TEXT-FIGURE 1.—Q_{O_2} time curves (30° C.) of slices of S91 melanoma washed in KR plus 10 percent PVP. *Upper group of curves.*—Run in KR (minus PVP) with 0.2 percent glucose and 0.005 *M* $KHCO_3$. A=control; A^1=plus coenzyme I (0.6 mg./ml.); B=plus succinate (0.03 *M*); B^1=plus succinate and coenzyme I. 2,4-DNP was tipped into A^1 and B^1, as indicated, to give a final concentration of 38 *gamma* per ml. *Lower group of curves.*—Same as *upper group*, but all vessels contained 10 percent PVP and 5 percent sucrose.

sucrose and coenzyme. Similar results were obtained in a number of experiments.

When tissue is very thinly sectioned, an increased rate with succinate may be obtained, but at the expense of the activity of other respiratory systems [*cf.* Rosenthal (7, p. 1716)]. This is well illustrated in the present study by results secured with normal mouse liver where separate and combined effects of excessive sectioning and of using an all-saline medium were noted (table 2). In KR, in the absence of added succinate, the Q_{O_2} of the finely cut liver tissue was lower than that of the same liver less finely sectioned. Decay in rate was most marked in the finely cut tissue (at the end of 74 minutes 27% *vs.* 8% decay). On the other hand, in the presence of 0.03 *M* succinate, the Q_{O_2} of the finely cut tissue was initially 30 percent higher than in the less finely cut tissue, but again the highest rate of decay occurred in the finely cut tissues (46% *vs.* 73% decay). The behavior of aliquots of the same 2 lots of tissue in MAS was in marked contrast to the behavior in KR. In MAS the basal rates without added succinate were initially higher than in KR (57% and 45% higher for large and small pieces, respectively). On the other hand, *just the reverse occurred in the presence of added succinate, i.e.,* the Q_{O_2} values in MAS were approximately 45 percent lower than in KR. However, as in KR, the decrease in rate was most rapid in the finely cut tissue. The results of this, and other similar experiments, show clearly that when the over-all integrity of the cellular metabolism is lowered by excessive sectioning of the tissue, and/or the use of an inorganic medium such as KR, the Q_{O_2} with exogenously supplied succinate is increased. While the effect of the endogenous metabolism in restraining succinate oxidation has long been recognized in attempts to maximize succinoxidase activity [*cf.* Rosenthal (7)] it has not always been taken into account in comparing the integrated oxidative metabolism of normal and malignant tissues. While reducing the thickness of the tissue section facilitates diffusion, it

TABLE 2.—*Respiratory activities of normal mouse liver sections (large vs. small pieces) at 38° C. in 2 different media with 0.2 percent glucose plus or minus 0.03 M succinate and with 100 percent oxygen in the gas phase*

Time at 38° C. (*minutes*)	Q_{O_2} in KR				Q_{O_2} MAS			
	−Succinate		+Succinate		−Succinate		+Succinate	
	Large* pieces	Small* pieces	Large pieces	Small pieces	Large pieces	Small pieces	Large pieces	Small pieces
6–24.....	6. 1	5. 6	21. 2	27. 5	9. 6	8. 1	11. 9	14. 7
(% *Succinate effect 6–24*)			(*247%*)	(*391%*)			(*24%*)	(*81%*)
24–39....	5. 6	4. 5	16. 4	17. 8	7. 2	6. 0	10. 8	11. 1
44–54....	5. 4	4. 3	14. 1	14. 0	7. 1	5. 4	10. 6	10. 0
54–74....	5. 6	4. 1	11. 4	10. 0	6. 7	5. 1	9. 6	9. 1

*The small pieces (mostly 0.5 to 1.0 mm. in cross section) were on the average about ¼ of the size of the large pieces, *i.e.*, with approximately 20 × the surface area. The tissue was washed once in *ca.* 20 volumes of MAS after sectioning. KR with 0.005 *M* $NaHCO_3$ had a *p*H of *ca.* 7.3, and MAS was adjusted to *p*H 6.8 with HCl. After 74 minutes' incubation with tissue, the *p*H values in KR were (minus and plus succinate, respectively) large pieces 8.0 and 8.2, small pieces 8.2 and 8.5. In MAS final *p*H values were 8.0–8.1 in all vessels.

introduces the important complications which result from a derangement of the endogenous metabolism.

The data show that under *in vitro conditions, which tend to maintain morphologic and physiologic integrity* (e.g., *in MS or MAS*), *the succinate responses of nongrowing normal, growing normal, and malignant tissues are all much more nearly alike than when comparisons are made under less physiologic conditions.* A careful distinction should be made in this connection between the comparative rates of oxygen consumption involving the operation of the whole Krebs cycle, and the rates occurring with a

TABLE 3.—*Respiratory activities of normal and malignant mouse tissues at 30° C. in various media with 0.2 percent glucose unless otherwise indicated**

Tissue and medium	Q_{O_2} at 30° C.		Succinate effect (*percent*)
	−Succinate	+Succinate (*0.03* M)	
Liver			
KR	2.1	12.0	471
KR plus 5% PVP	3.6	10.8	200
Earle's medium	5.0	12.6	152
MS	3.3	6.0	81
Kidney			
KR	8.6	14.1	64
KR plus coenzyme I	9.4	12.3	30
Horse serum†	11.9	16.0	34
MS†	11.1	15.0	35
S91 melanoma			
KR	3.2	9.2	188
KR plus 5% PVP	3.9	8.8	126
KR plus 10% PVP	3.3	6.1	85
KR plus coenzyme I	3.8	5.6	47
KR plus 5% PVP plus coenzyme I	4.6	5.7	24
KR plus 10% PVP plus coenzyme I	3.9	5.9	51
Earle's medium	4.2	7.5	78
Earle's plus coenzyme I	4.5	6.5	44
MAS† (tissue separated without sectioning)	5.2	5.7	10
Krebs-2 ascites tumor			
KR‡	3.6	3.6	0
MAS†	4.6	4.6	0

*Brackets designate data of individual experiments involving the same tissue.
† No added glucose.
‡ 0.1% glucose.

virtually unrestrained succinic oxidase system, as with exogenous succinate and very thin slices or homogenates. In the latter cases, certain normal adult tissues may show exceedingly high rates of oxygen consumption, *e.g.*, $Q_{O_2}=100$ in liver (*7*).

The foregoing observations indicate that a large *in vitro* response to succinate can be due in large measure to tissue alteration resulting from conditions incident to tissue preparation and subsequent incubation. Such effects are most marked in a tissue like liver (tables 1, 2, and 3). The data show that under conditions which tend to maintain cellular integrity (*e.g.*, in

MAS) the steady-rate Q_{O_2} without added succinate tends to be relatively higher, and the Q_{O_2} with added succinate relatively lower, than in plain KR. The fact that addition of PVP to KR tends to act in the same direction as the addition of coenzyme I (text-fig. 1 and table 3) might indicate that maintenance of nuclear integrity is involved, since Hogeboom and Schneider (*8*) have shown that coenzyme I synthesis is localized in the nuclei, and PVP-sucrose solutions tend to stabilize the nuclei (*9*).

Effect of 2,4-dinitrophenol.—With tissues which have a considerable hexokinase activity it is possible to obtain marked increases in the rates of both respiration and aerobic acid formation in the presence of glucose by addition of proper concentrations of the "uncoupling" (*10*, p. 67) agent 2,4-DNP. For example at 30° C. kidney slices in MS had a Q_{O_2} of 11.1 without added succinate and 15.0 with added succinate (*ca.* 0.03 *M*). Following addition of 38 p.p.m. 2,4-DNP the Q_{O_2} values became 17.0 and 17.6, respectively. Similar results were obtained with brain, embryo head, S91 melanoma, and Krebs-2 ascites tumor. In some, but not all, cases Q_{O_2} values with 2,4-DNP were higher in the presence of added succinate, or other Krebs-cycle acid, than in their absence. With excessive concentrations of 2,4-DNP (76 p.p.m., more or less, depending on the medium and tissue) the respiration of the cells was lowered because of injury. MS or MAS gave marked protection against the "uncoupling" action of 2,4-DNP; generally, in the serums about double the concentration was required to be as effective as that in KR or KR plus PVP. Used in this way, 2,4-DNP permits evaluation of the degree of respiratory restraint operating in a tissue by indicating the extent to which this can be reversed. While this restraint may result from a lack of available phosphate acceptors, the action of DNP is such that a rapid turnover of phosphate takes place, so that oxidation via the Krebs cycle is greatly accelerated (*10*).

Table 4 compares the steady-rate Q_{O_2} values of S91 melanoma and brain slices from the same animals, in KR plus 10 percent PVP at 38° C., with the maximum Q_{O_2} values attained after adding 2,4-DNP. The highest values reached were very similar in the 2 tissues, *i.e.*, 17.1 in melanoma and 18.3 in brain. The increases due to 2,4-DNP were slightly

TABLE 4.—*Respiratory activities of S91 melanoma and brain slices from the same animals in KR plus 10 percent PVP at 38° C. with 0.2 percent glucose plus or minus 0.03* M *succinate, before and after addition of 2,4-DNP*

Measurement	S91 melanoma Q_{O_2}		Brain Q_{O_2}	
	−succinate	+succinate	−succinate	+succinate
Q_{O_2} before 2,4-DNP	7.0	12.0	10.0	14.2
Max. Q_{O_2} after 2,4-DNP*	13.9	17.1	15.7	18.3
$\Delta Q_{O_2} \pm$ 2,4-DNP	6.9	5.1	5.7	4.1
*p*H change†				
Minus 2,4-DNP	−1.03	−0.97	−0.85	−1.05
Plus 2,4-DNP	−1.17	−1.23	−1.33	−1.23

*Thirty-nine *gamma* per ml. after tipping.
†Final *p*H without tissue = 8.85.

TABLE 5.—*Respiratory activities of Krebs-2 ascites-tumor cells and washed brain slices of the same animals at 38° C. with glucose 0.2 percent plus or minus 0.03 M succinate, and washed slices of embryo head in Earle's medium or KR with 0.3 percent glucose*

Measurement and medium	Krebs-2 ascites tumor Q_{O_2}		Brain Q_{O_2}		Embryo head (13 days old) Q_{O_2}	
	−succinate	+succinate	−succinate	+succinate	−succinate	+succinate
Q_{O_2} before 2,4-DNP						
MAS	7.9	7.9	9.5	14.8	9.6 Earle's medium	11.4
KR plus 5% PVP	5.9	5.9	8.2	11.1	8.6 KR	10.0
Max. Q_{O_2} after tipping 2,4-DNP						
MAS	13.4*	16.4*	11.1†	16.0†	17.1* Earle's medium	18.1*
KR plus 5% PVP	19.7†	22.6†	12.8†	14.8†	12.5† KR	17.5†
ΔQ_{O_2}+2,4-DNP						
MAS	5.5	8.5	1.6	1.2	7.5 Earle's medium	6.7
KR plus 5% PVP	13.8	16.7	4.6	3.7	3.9 KR	7.5
pH *change*‡						
MAS	−1.11	−1.30	−1.00	−1.10	—	—
MAS plus 2,4-DNP	−1.22	−1.50	−1.25	−1.25	—	—
KR plus 5% PVP	−1.96	−1.87	−0.97	−1.02	−1.22 Earle's medium +2,4-DNP	−1.25
KR plus 2,4-DNP	−3.44	−2.42	−1.47	−1.44	−1.82 KR +2,4-DNP	−1.66

* Seventy-eight *gamma* 2,4-DNP per ml.
† Thirty-nine *gamma* 2,4-DNP per ml.
‡ Final pH of MAS without tissue was 0.30; of KR plus 5% PVP, 8.05; of Earle's medium, 9.21; and of KR, 8.80.

greater in the S91 melanoma than in brain. Table 5 makes a similar comparison between Krebs-2 ascites cells and brain tissue from the same animals together with 13-day-old embryo head. The increases due to 2,4-DNP were considerably larger in the tumor and embryonic tissue than in the normal adult tissue. The highest maximum Q_{O_2} values were in the ascites cells. The greatest resistance to "uncoupling" was also shown by the ascites cells. These data are in harmony with the view that in Krebs-2 ascites tumor the respiration is strongly restrained and relatively stable under *in vitro* conditions. A number of experiments gave similar results.

That the high rates of respiration in "uncoupled" Krebs-2 cells did not involve merely the succinic oxidase system is shown by table 6. The maximum Q_{O_2} with glucose as the only added substrate was nearly the same as with succinate plus glucose (20.2 *vs.* 22.5). Furthermore, succinate, malate, pyruvate, and citrate were metabolized, as evidenced by the fact that when the Q_{O_2} with glucose alone had fallen to 1.0 the rates with the other substrates also present were in the range 8.6 to 14.2. This result was not due to exhaustion of glucose, but rather resulted from a protecting

TABLE 6.—*Effect of 2,4-DNP (39 gamma/ml.) on Q_{O_2} of Krebs-2 ascites-tumor cells in KR plus 5 percent PVP at 38° C.*

Substrates* added	Q_{O_2} before 2,4-DNP	Max. Q_{O_2} with 2,4-DNP	Q_{O_2} 46 min. after tipping 2,4-DNP	Final *p*H
Glucose only	5. 8	20. 2	1. 0	5. 28
Succinate	6. 1	22. 5	14. 2	6. 25
Malate	6. 1	24. 9	8. 6	5. 78
Pyruvate	5. 9	20. 2	9. 4	5. 60
Citrate	5. 6	22. 1	12. 3	6. 12

*Initial glucose concentration of 0.2 % in all vessels, all other substrates 0.03 *M* (equivalent to anhydrous acids).

*p*H effect occasioned by release of alkali during metabolism of the neutral sodium salts of the respective acids (table 6).

Effect of oxygen deficiency.—Slices of normal adult mouse brain were incubated in KR at 38° C. with either 100 percent oxygen or nitrogen in the gas phase. Following this preincubation the vessels were removed from the bath, NaOH was added to the center wells, and the system re-gassed with 100 percent oxygen. Table 7 shows the effects of preincubation for 30 minutes in the presence of 0.2 percent glucose. Under these conditions glycolysis occurred both in oxygen and nitrogen, although the rate in the latter fell off with time. After adding NaOH it was immediately apparent that the respiration of the tissue preincubated in nitrogen had been damaged. The succinic oxidase system, while damaged, was injured less than the total respiration (lowered 24 and 43%, respectively). This of course gave a large increase in the succinate effect. The marked lowering of *p*H by the tissue preincubated in nitrogen showed that little or no damage had occurred to the glycolytic system. However, whereas 2,4-DNP increased

TABLE 7.—*Effect on metabolism of preincubating washed slices of normal adult brain in KR with 0.2 percent glucose in 100 percent oxygen or nitrogen at 38° C. for 30 minutes (NaOH added to center wells and re-gassed with O_2 following preincubation)*

Measurement	Tissue preincubated 30′ in O_2			Tissue preincubated 30′ in N_2		
	Q_{O_2} −succinate	Q_{O_2} +succinate	Succinate effect (*percent*)	Q_{O_2} −succinate	Q_{O_2} +succinate	Succinate effect (*percent*)
Before adding 2,4-DNP..	7. 5	14. 1	88	4. 3	10. 8	151
After adding 2,4-DNP*..	11. 7	16. 8	43	3. 0	9. 6	220
ΔQ_{O_2}.....	4. 2	2. 7	—	−1. 3	−1. 2	—
*p*H change (*p*H without tissue=8.71).....	−2. 21	−1. 97	—	−2. 62	−2. 07	—

*Final 2,4-DNP concentration was 37 *gamma* per ml.

the Q_{O_2} of tissue preincubated in oxygen, it did not do so in the tissue preincubated in nitrogen.

Table 8 illustrates more drastic injury obtained in an experiment similar to the one shown in table 7 with the difference that preincubation was carried out for 60 minutes in the absence of glucose. Again, a markedly differential response of the basic respiration (absence of added succinate) and the succinic oxidase system was obtained. Here, however, the glycolytic system was markedly damaged. Again no significant stimulation of respiration occurred following addition of 2,4-DNP. These experiments show, as would be expected (*1,2*), that exposure of normal adult tissue to lack of oxygen results in a lowering of respiratory capacity (tables 7 and 8), but in marked contrast to the low respiration of tumors (tables 4, 5, and 6), this is not reversed by 2,4-DNP. Another point of difference is to be found in the large succinate effect in tissue damaged by exposure to anaerobic conditions, whereas in tumors, or certain growing normal tissues, the response to succinate is minimal.

TABLE 8.—*Effect on metabolism of preincubating unwashed slices of normal adult brain in KR without glucose in 100 percent oxygen or nitrogen at 38° C. for 60 minutes (NaOH was added to center wells and vessels re-gassed with O_2 following preincubation)*

Measurement	Tissue preincubated 60′ in O_2			Tissue preincubated 60′ in N_2		
	Q_{O_2} −succinate	Q_{O_2} +succinate	Succinate effect (*percent*)	Q_{O_2} −succinate	Q_{O_2} +succinate	Succinate effect (*percent*)
Before adding glucose or succinate.....	4. 2	—	—	1. 2	—	—
After adding glucose*± succinate.....	3. 9	13. 8	254	0. 23	10. 1	3391
After adding 2,4-DNP†..	7. 2	15. 0	108	0. 53	7. 4	1296
$\Delta Q_{O_2} \pm$ 2,4-DNP.....	3. 3	1. 2	—	0. 30	−2. 7	—
*p*H change (final *p*H without tissue=9.18)..	−1. 48	−1. 43	—	−0. 46	−0. 45	—

*Final glucose concentration was 0.2% and succinate 0.04 *M*.
†Final 2,4-DNP concentration was 54 *gamma* per ml.

Discussion

Greenstein (*5*) has noted that there is a certain overlapping of the values for succinate response of normal and malignant tissues. As the result of his own researches and those of others(*5*, p. 270) he came to the significant conclusion that " . . . the equilibrium position of cytochrome c in normal tissues lies further to the oxidized side than it does in malignant tissues." From this, Greenstein drew the implication that the O/R potential of malignant tumors lies nearer to the reducing or anaerobic end of the scale than it does in normal tissues—a view consistent with the large glycolytic capacity of tumors. In the present investigation observations of both malignant and nonmalignant tissues indicate that a large *in vitro* increase in the succinate effect can result from tissue modification during preparatory and incubation procedures. It seems possible that the differing *in vitro* respiratory stabilities of nongrowing normal and malignant tissues in this respect may be related to the differences in O/R balance elucidated by Greenstein *et al.* (*5*). In this connection it should be noted that certain growing nonmalignant tissues (chorion, embryo head), like tumors, show little response to exogenous succinate. Such normal tissues possess a powerful glycolytic system, which, as in growing tumors, might be involved in their behavior towards succinate.

The steady-rate Q_{O_2} values of Krebs-2 ascites cells are certainly low (5.0–10.0) in relation to their high anaerobic glycolytic capacities ($Q^{N_2}_{CO_2}$=50–100). In such a tumor the relatively low Q_{O_2} values could be expected on the basis of an irreversibly damaged respiration. However, there are several lines of evidence which indicate that the relatively low Q_{O_2} of the Krebs-2 ascites tumor is not due to an actual loss of respiratory potential of the type which results from a partial destruction of respiratory capacity, *e.g.*, by exposure to a lack of oxygen.

Chance (*11*) has shown by direct spectrophotometric determinations that intact ascites-tumor cells contain amounts of cytochrome c, other respiratory pigments, and dehydrogenase activities that are in the range of normal tissues. In the present investigation release of respiratory restraint by 2,4-DNP resulted in Q_{O_2} values in both Krebs-2 and S91 tumors that were well in the range of nonmalignant tissues compared under the same conditions. That such increases were not due merely to succinoxidase activity was evidenced by high Q_{O_2} values obtained with glucose alone, or glucose plus organic acids other than succinate. While exposure of normal adult tissue to lack of oxygen resulted in a lowering of respiration, this was not reversed by 2,4-DNP. Clowes and Keltch (*12*) have recently reported that dinitrocresol increased oxygen consumption by Ehrlich ascites cells, and it has long been known [*cf.* Burk (*13*)] that dinitrophenols can increase the respiration of both tumor and normal cells. Furthermore, exposure of normal tissue to lack of oxygen (tables 7 and 8) resulted in a relative increase in the succinoxidase excess over the intermediary metabolism—a situation just the reverse of that found in tumors. It is important to note in this connection that undamaged

(with respect to exposure to anaerobic conditions) normal-growing tissues behaved toward exogenous succinate like tumors.

It has recently been shown experimentally that the glycolysis of certain tumors is under variable restraint by a stress-modifiable anti-insulin hormonal mechanism (*3,4*). In a tumor like the S91 melanoma, glucose utilization is quite strongly under the control of this anti-insulin system. In the derived amelanotic S91A tumor, representing a further degree of anaplasia, sensitivity to anti-insulin regulation of glucose metabolism is markedly weakened, and in the Krebs-2 ascites (or solid) tumor almost all sensitivity to hormonal restraint is gone. The capacity of the host to affect the tumor glycolysis (as measured subsequently *in vitro*) is paralleled by the host's ability to restrain growth of the tumor *in vivo* under stressful conditions (*3,4,14*).

If the development of malignant cells depends (in part) on a loss of sensitivity to the anti-insulin hormones which regulate glucose phosphorylation, it would be expected on the basis of the work of Crabtree (*15*), Rosenthal *et al.* (*16*), and others that the Q_{O_2} of such cells would be lowered as the loss of sensitivity to the glycolysis-restraining hormones became greater. However, it is clear that the actual functioning of glycolysis *per se*, cannot be the only cause of a restrained respiration in the S91 melanoma or in the Krebs-2 ascites tumor, since elimination of glucose from the incubation medium increases the respiration by only 15 to 30 percent. The shifting of the intracellular O/R potential toward the reducing or anaerobic side, as suggested by Greenstein (*5*), may well play a major role in lowering the respiratory rate of the malignant cell. In any case, however, cells in which the anti-insulin hormonal restraint of glucose phosphorylation is lowered should be adapted to survival in an anaerobic environment, where the factors of natural selection would operate as outlined by Warburg (*2*) to further increase the glycolytic capacity. Any of the respiration-inhibiting agents mentioned by Warburg (*2*) could similarly promote selection in the direction of an increasingly greater glycolytic metabolism.

Consideration of the possible role of mutant, or virus-modified, mitochondria (*17,18*) in the determination of the shifted metabolic balance characteristic of the cancer cell is emphasized by the findings of duBuy *et al.* (*19*) and Hesselbach and duBuy (*20*) that mitochondria play a role in glycolysis that is more important than has been generally recognized.

Summary

When comparisons were made in mouse ascitic serum, or in normal mouse serum, the steady-rate Q_{O_2} values of Krebs-2 ascites cells and S91 melanoma slices were lower than in slices of kidney, liver, brain, or chorion. Q_{O_2} values of 12- to 14-day-old embryonic head tissues were only slightly higher than those of S91 melanoma. Q_{O_2} values of all tissues in Krebs-Ringer solution were generally lower (*ca.* 15 to 50%) than in mouse ascitic serum or normal serum, especially in the case of liver.

The percentage response to succinate in Krebs-Ringer was, in order of increasing magnitude, Krebs-2 ascites, embryo head, S91 melanoma, brain (or kidney), and liver. In ascitic serum the Q_{O_2} values without added succinate were relatively higher, and the values with succinate tended to be relatively lower, than in Krebs-Ringer solution, so that the percentage succinate effect was markedly reduced.

The addition of coenzyme I to Krebs-Ringer solution markedly lowered the response to succinate but slightly increased the Q_{O_2} without added succinate. The addition of 5 to 10 percent polyvinylpyrrolidone (PVP), or PVP plus sucrose, gave results similar to those produced by addition of coenzyme. The data indicate that large increases in the succinate effect can result from tissue modification resulting from preparatory and incubation procedures. Such an artefact is most pronounced in an unstable (under *in vitro* conditions) tissue like liver and minimal in the relatively stable Krebs-2 ascites cells.

Exposure of both malignant and nonmalignant tissues to the "uncoupling" agent 2,4-dinitrophenol resulted in respiratory stimulation of such a character that the tumor Q_{O_2} values rose to well within the range of nonmalignant tissues compared under the same conditions. When slices of normal brain were exposed to anaerobic conditions, the respiration measured subsequently in oxygen was markedly decreased. In contrast to tumors, the oxygen consumption of such cells could be greatly increased by exogenous succinate, but not by the addition of 2,4-DNP. The data are more consistent with a concept of respiratory restraint in cancer than with a concept of an irreversibly damaged respiration.

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Combined Treatment of Advanced Leukemia (L1210) in Mice with Amethopterin and 6-Mercaptopurine[1,2]

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The employment of combinations of drugs for treatment of malignant disease has been given considerable attention in recent years (*1–25*). In general, demonstrations of synergism have resulted from studies on the effect of combined treatment on tumor growth alone. However, the therapeutic usefulness of an additive or synergistic effect of combined treatment against tumor growth may be limited by the increased toxicity of the combination of drugs for the host. This was illustrated in an earlier investigation (*9*), where it was observed that treatment of leukemia L1210 in mice with certain combinations of amethopterin and 6-mercaptopurine (6-MP) did not provide synergistic therapies. Whatever synergism there may have been with respect to anti-tumor effect was negated by the enhanced toxicity of combined treatment. Treatment with amethopterin alone proved a better therapy than 6-MP alone or any of the combined treatments.

In the prior investigation (*9*) only a single treatment with the drugs was employed, and further, this treatment was administered early in the course of the disease. The possibility remained that with some other treatment schedule the 2 drugs could still provide a synergistic therapy.

In the treatment of leukemia in mice, attention has been focused on the employment of multiple-treatment schedules. Previous studies have indicated that for both amethopterin and 6-MP administered individually, such schedules are more effective than single treatment in extending the survival time of leukemic mice (*26,27*). Also, there has been considerable interest in studies involving the treatment of leukemia late in the course of the disease. Such studies became practical with the demonstration that daily treatment with either amethopterin (*28*) or 6-MP (*29*) gave significant increases in the survival time of mice bearing advanced leukemia. In view of the relatively successful treatment of early leukemia by appropriate alterations of schedules of drug administration (*30,31*),

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it was felt that such modifications might also increase the effectiveness of treatments against advanced leukemia.

The current experiment was conducted to investigate the influence on advanced leukemia of 2 types of multiple-treatment schedules employing amethopterin and 6-MP, alone and in combination.

Methods

General.—The general procedures employed have been previously described (*28*). Ten-week-old CDBA hybrid [4] male mice were inoculated in the right thigh with 0.1 ml. of a uniform saline suspension (17×10^6 cells per ml.) of leukemic cells (L1210) taken from DBA/2J mice. In order to test the potency of the inoculum, the leukemic mash was serially diluted and inoculated into CDBA male mice. It was thus demonstrated that the experimental and control animals received a concentration of tumor cells which was 100 times the concentration that resulted in 100 percent lethality in untreated mice.

Amethopterin [5] was dissolved in 2 percent sodium bicarbonate and 6-MP [6] was dissolved in dilute sodium hydroxide. The solution of 6-MP was brought to *p*H 8 to 9 with dilute hydrochloric acid. The drugs were administered intraperitoneally in a constant volume of 0.01 ml. per gm. of body weight. The mice were randomized into prescribed injection groups of 8 mice each.

Experimental.—Two schedules of combined therapy were employed. For one schedule both amethopterin and 6-MP were administered daily. For the second combined schedule 6-MP was given daily and amethopterin was administered every 3rd day. When administered on the same day, the drugs were injected concomitantly. In addition, corresponding schedules with each drug administered alone were employed. For each schedule of therapy a series of dose levels was employed. Because of the sharp increase in amethopterin toxicity on repeated administration, relatively small amounts of amethopterin were administered in the combined schedules. When amethopterin was administered daily with 6-MP the relative dosage was 1 part amethopterin to 36 parts 6-MP. When amethopterin was administered every 3rd day, with 6-MP daily, the reduced toxicity of amethopterin permitted an increase in the relative dose of amethopterin to 6-MP. The ratio employed was approximately 1 part amethopterin to 7.7 parts 6-MP (or about 1 to 23 in terms of average daily dose). In the previous experiment, where multiple-treatment schedules were not employed, the minimum ratio of amethopterin to 6-MP had been 1 to 7.5 (*9*).

In order to insure that the drugs would challenge a frankly systemic leukemia, treatment was initiated on the 8th day following leukemic

[4] (BALB/cAn × DBA/2J)F_1.

[5] Amethopterin was kindly provided by Dr. J. M. Ruegsegger, of the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, N. Y.

[6] 6-Mercaptopurine was kindly provided by Dr. George Hitchings, of the Wellcome Research Laboratories, Tuckahoe, N. Y.

inoculation, at which time all the mice displayed large, palpable local tumors at the site of leukemic inoculation. Treatment was continued until all the animals succumbed.

On the initial day of treatment, 10 leukemic mice were selected at random and the capacity of the blood and of the spleen to transmit the disease to other CDBA mice was tested. In every case the transfer of blood and of spleen mash resulted in leukemic growth and eventual death.

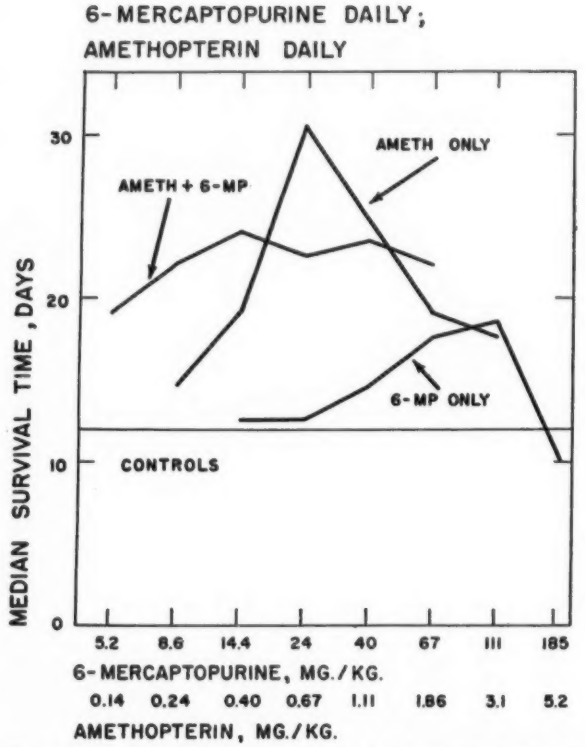
Evaluation procedure.—In the previous investigation, in which single treatment early in the course of the disease was employed, a temporal separation of toxic and tumor deaths resulted, and it was possible to compare alternative therapies in terms of their antileukemic specificities (*9*). In the current investigation, involving multiple-treatment schedules, no clear temporal separation could be made. Consequently, the earlier method of comparing alternative therapies was not feasible. Instead, the various therapies were compared by the employment of an assay procedure applicable to the study of the treatment of advanced tumor. This assay procedure has been previously discussed in detail (*28*). For each therapy a series of dose levels is employed and the optimal dose for the therapy is considered to be that dose at which the maximum survival time is observed. Alternative therapies are then compared on the basis of their respective maximum survival times.

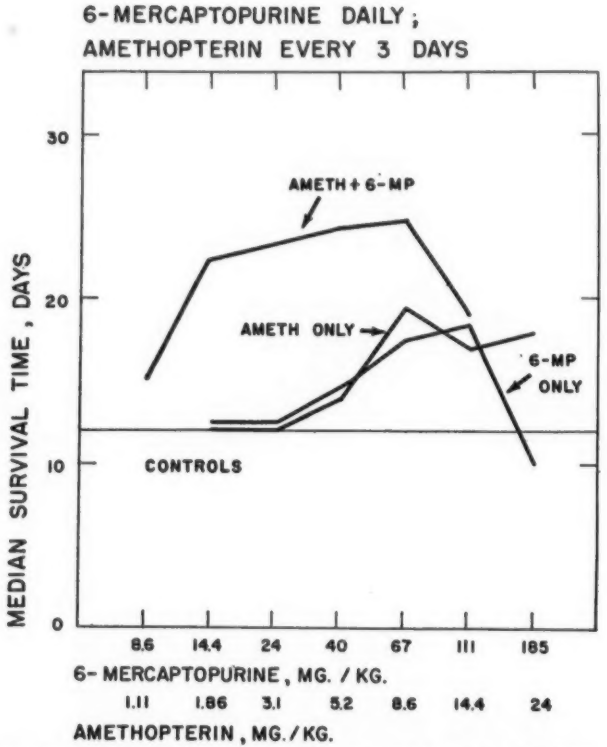
Results

The results of this experiment are summarized in text-figure 1, which shows the median survival time at each dose level for each of the treatment schedules. It may be noted that for each treatment employed there is an optimal dose level which elicits the greatest prolongation of survival time. At lower doses, the treatments were less effective and the mice succumbed with large local tumors. At higher doses, the mice lost weight soon after treatment was initiated and succumbed from drug toxicity with small or no local tumors.

The *left* panel of text-figure 1 represents the results obtained, employing daily doses of both amethopterin and 6-MP. At its most effective level, treatment with amethopterin alone raised the median survival time from 12 (± 0.4) days, for untreated mice, to 30.5 (±2.0) days. In contrast, treatment with 6-MP alone elicited a maximum median survival time of only 18.5 (±0.8) days, while combined treatment yielded a maximum median survival time of 24 (±0.9) days.[7] All these extensions of survival time are noteworthy, especially when it is considered that treatment was initiated only 4 days prior to the median time of death of untreated mice. However, the relative advantage of treatment with amethopterin alone at a suitably selected dose level is apparent. This result is in

[7] The standard errors were estimated from the range, according to the method of Mantel (*38*). The survival time of the mice treated with amethopterin alone at 0.67 mg. per kg. is significantly greater than the survival time at the optimal combined treatment. It is noteworthy that while only 1 of 8 mice receiving 0.67 mg. per kg. amethopterin alone died prior to day 28, all the mice on the optimal combined treatment were dead before day 25, and all the mice at the optimal dose of 6-MP alone were dead before day 22. The standard errors shown do not reflect this but rather give undue weight to the occasional animal dying extremely early or late.





TEXT-FIGURE 1.—Effect of treatment of advanced leukemia L1210 with amethopterin and 6-mercaptopurine (6-MP), separately and in combination. Control results and results for treatment with 6-MP alone are repeated in the 2 panels of the figure. Median survival times are based on 8 mice in each treatment group and on 18 mice for the controls.

agreement with the earlier findings (*9*) where amethopterin and 6-MP were administered alone and in combination as single treatments early in the course of the disease. Amethopterin alone was the most effective treatment.

The *right* panel of text-figure 1 shows the results obtained by administering amethopterin every 3rd day and 6-MP daily. Amethopterin alone yielded a maximum median survival time of 19.5 (±2.0) days, which was just slightly in excess of the 18.5 days for daily 6-MP alone. With combined treatment the maximum median survival time was raised to 25 (±1.4) days; in addition, 3 other levels of combined treatment yielded a greater median survival time than the maximum of 19.5 days obtained with amethopterin alone. In this case, it is clear that combined treatment is superior to treatment with either drug alone.

Discussion

For a given schedule of treatment, a synergistic therapy for 2 drugs may be said to exist when combined treatment at the optimal level provides a more effective therapy than can be obtained with either drug alone.[8] Where, as in the present case, the usefulness of a treatment is limited by its toxicity for the host, therapeutic synergism requires that the anti-tumor effects of the separate drugs combine to a greater extent than do their toxic effects.

In the present investigation the demonstration of synergism between amethopterin and 6-MP was dependent on the schedule of treatment employed. When each drug was administered daily there were several dose levels at which the combined treatment was superior to treatment with the corresponding dose levels of the individual drugs (text-fig. 1, *left* panel). However, when comparisons were made at the optimal dose level, combined treatment proved inferior to treatment with amethopterin alone. On this schedule, the increased anti-tumor effect of combined treatment was negated by the increased toxic effect for the host. In contrast, when amethopterin was administered every 3rd day and 6-MP daily (text-fig. 1, *right* panel), combined treatment was superior to either drug separately—both at the individual dose levels and at the optimal dose level. In this case, the increased anti-tumor effect for the combination was retained.

Comparison of the 2 panels of text-figure 1 shows that the decrease in the frequency of amethopterin treatment had little influence on the effect of combined therapy but sharply reduced the effectiveness of amethopterin when employed alone. The latter observation is in agreement with the

based on 8 mice in each treatment group and on 18 mice for the controls.

[8] The term "synergism" is used here in its broad sense as meaning the cooperation or effective combination of agents. This definition permits the user to specify the nature of the cooperation with which he is concerned. In particular, we have been concerned with the combined action of agents which results in improved therapies. This form of cooperation we are calling therapeutic synergism. Investigators have applied the term "synergism" to other forms of cooperation in which they have been interested without appropriately qualifying the term. Frequently these other forms of synergism have no implication with respect to improved therapy; sometimes they may result only in more economical rather than in improved therapies. To the extent that reported findings of synergism are frequently considered for therapeutic application, the authors feel that the nature of cooperation found and the evidence for resulting improvement in therapy should be made clear.

previous report (*28*) that when treatment is initiated late in the course of the disease daily treatment is superior to less frequent treatment. It is likely that the beneficial effect of combined treatment (amethopterin every 3rd day; 6-MP daily), as compared to treatment with amethopterin alone every 3rd day, results from the shorter time interval between antileukemic treatments.

It should be noted that, despite the demonstration of synergism employing daily treatment with 6-MP combined with amethopterin every 3 days, this combined treatment still was less effective than amethopterin alone administered daily. Thus, the most advantageous therapy observed in this experiment was still one requiring treatment with only a single drug. The synergism found is specific for its treatment schedule and does not necessarily apply to other schedules. This suggests that, in general, demonstrations of synergism had best be interpreted with caution. Synergism between drugs may not be sustained if there is any important modification in the method of application. Conversely, failure to find synergism may result from the methods of application used. Of significance, also, is the possibility that a demonstrated synergism may fail to be therapeutically useful when, on some other schedule of treatment, employment of a single drug is more beneficial.

Studies involving the host-tumor-drug relationship have shown that anti-tumor specificity of action is not a fixed property of the drug alone but may be influenced by a number of factors including: the stage in the course of tumor growth when treatment is initiated; the number and spacing of treatments; the use of metabolites with antimetabolites; the age and weight of the test animals; and the concentration of the implanted tumor inoculum (*7,26,30,31,33–36*). The results of the present experiment indicate that the relative effectiveness of combined treatment, also, is not fixed but is dependent on factors which tend to alter the host-tumor-drug relationship.

Summary

The present experiment represents an evaluation of the effectiveness of 2 combined-treatment schedules employing amethopterin and 6-mercaptopurine against advanced leukemia (L1210) in mice. When both drugs were administered daily, the most effective level of the combined treatment was inferior to the most effective level of amethopterin alone in increasing the survival time of the mice. When amethopterin was given every 3rd day and 6-mercaptopurine was given daily, the combined treatment was more effective at several dose levels than the most effective level of either drug alone. This combined treatment, however, was less effective than optimal treatment with amethopterin alone administered daily.

The data indicate that the demonstration of synergistic action between 2 drugs is dependent on the schedule of treatment employed. Further, it is suggested that a demonstrated synergism for a particular schedule of treatment may have little significance when, on some other schedule, treatment with a single drug is superior.

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Subcutaneous Fibrosarcomas in Strains C3H and C57BL Female Mice, and F_1 and Backcross Hybrids of these Strains [1]

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Numerous reports have been published concerning subcutaneous sarcomas induced in mice by a variety of agents (*1*), but very few reports can be found describing sarcomas in the subcutaneous tissues of untreated mice. Guérin (*2*) in 1954 published a review of spontaneous tumors in mice. He listed preceding reports of sarcomas and stated that it was very rare to see in mice a pure fusiform sarcoma composed of young fibroblasts having a fascicular pattern. He found only 4 among 6,000 mice autopsied from his colony. Cloudman (*3*) in 1941 stated that sarcomas were among the more common malignant tumors in the subcutaneous regions, but there was no high-tumor stock, and it was unusual to find an incidence of over 15 percent. He gave illustrations of the more common histologic forms and noted that many of the tumors showed polyhedral cells. Slye, Holmes, and Wells (*4*) in 1917 gave the most complete account of a large number of subcutaneous sarcomas. They reviewed preceding descriptions of 17 tumors and reported 87 of their own which were found among 12,000 mice autopsied from the Slye colonies.

A large number of subcutaneous tumors was found by us in a recently completed study of strain C3H with the mammary tumor agent, C3Hf lacking the agent, C57BL female mice, and F_1 and backcross hybrids of these strains. This was an extensive study that was begun in 1946 and extended over the succeeding 8 years. It included a total of 4,049 mice that were allowed to live their normal life span, and were killed only when they appeared to be in poor health or had palpable tumors. The tumors were not allowed to attain a large size, and usually measured 1 to 1.5 cm. in diameter. All the mice were autopsied and all grossly observed lesions were taken for histologic study. All the mice were females, since the primary purpose was to study the control of the gene or genes over the mammary tumor agent in the development of mammary-gland tumors (*5*).

One hundred and forty-six subcutaneous tumors, excluding mammary-gland tumors, were found. This number is sufficiently large to furnish significant information as to the incidence of subcutaneous neoplasms

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in these 2 strains and their hybrids, the age at which the mice developed the neoplasms, the location, coexistence with other neoplasms, and histologic features. The primary purpose of this report is to present an analysis of the tumors with respect to these points. In addition, however, information on the occurrence of these tumors in the large number of C3H and C3Hf females is of value in properly judging the significance of reports on the induction of subcutaneous fibrosarcomas in C3Hf mice treated when newborn with a variety of cell-free filtrates (*6,7*). Such incidence data may also be of value in considering the significance of the malignant change which developed in fibroblasts grown *in vitro*. These were first obtained from the subcutaneous tissues of strain C3H mice, and then grown in tissue culture with and without exposure to methylcholanthrene for varying periods of time. On injection into strain C3H mice, many of the cultures produced fibrosarcomas at the site of inoculation (*8,9*).

Observations

Number.—A total of 788 subcutaneous neoplasms was found in the 4,049 mice of the present series. Of these, 642 were mammary tumors, 23 were hemangioendotheliomas, 8 were osteogenic sarcomas, 1 was a myoepithelioma, and 3 were reticulum-cell neoplasms, Type A. The histology of the last 3 types of tumors has been described (*10–12*). No pleomorphic tumors of the parotid gland were found. Five sarcomas were thought to have originated from the mammary gland. The present report will deal with 106 tumors composed of fusiform cells within the subcutaneous tissues, and presumably of fibroblastic origin. These formed a large majority of all subcutaneous growths. Descriptions of other neoplasms and unusual lesions found in these mice are planned for future publications.

Six sarcomas of a similar histologic type but not in subcutaneous tissues were located as follows: pancreatic area, mediastinum, attached to the peritoneal surface of the abdominal wall, base of the brain, mesenteric lymph node, and neck of the bladder. A few sarcomas of similar histology were also found in the uterus, and these will be described separately with other tumors of this organ.

Relation to genotype of mouse.—The 106 subcutaneous sarcomas are listed in table 1 according to the genetic group in which they occurred. All were found in C3H, C3Hf, or hybrid females that contained at least 50 percent C3H chromatin, *i.e.*, the F_1 and C3H backcross hybrids. None occurred in the 2 groups of C57BL females or in the C57BL backcross females. Thus, the C3H genotype is relatively susceptible to this type of neoplasm, whereas the C57BL genotype is resistant. In 1938, Andervont reported the induction of subcutaneous sarcomas in these and other strains with methylcholanthrene and dibenzanthracene (*13*). It is interesting that, although the numbers that could be compared directly were small in his groups of C57BL and C3H mice, the percentage of C3H mice developing sarcomas was higher than that of the C57BL mice.

TABLE 1.—*Incidence of subcutaneous sarcomas in various genetic groups**

Group	Number of animals	Average age at death (*months*)	Number with sarcomas	Average age when tumor occurred (*months*)
C3H	20	10.1	0	—
FN†–C3Hf	88	11.7	0	—
(C3H × B‡)F_1	90	16.4	0	—
FN–C3Hf	80	14.9	0	—
(C3HB × B)BC_1	82	21.3	0	—
FN–C3Hf	69	15.0	0	—
(C3HB × B)BC_2	73	23.2	0	—
FN–C3Hf	97	20.7	3	24.7
(C3HB × B)BC_3	96	22.8	0	—
FN–C3Hf	93	19.7	1	25.0
(C3HB × B)BC_4	84	21.4	0	—
FN–C3Hf	89	16.3	0	—
(C3HB × B)BC_5	87	21.8	0	—
FN–C3Hf	76	23.4	8	27.1
(C3HB × B)BC_6	60	23.1	0	—
FN–C3Hf	89	24.2	9	25.8
(C3HB × B)BC_7	78	23.9	0	—
(BBC_7 × C3Hf)F_1	100	27.7	3	31.0
(BBC_7C3Hf × C3Hf)BC_1	100	24.7	4	27.8
(BBC_7C3Hf × C3Hf)BC_2	96	23.0	2	23.5
(BBC_7C3Hf × C3Hf)BC_3	100	21.3	3	28.0
(BBC_7C3Hf × C3Hf)BC_4	103	21.8	4	23.8
C57BL	17	22.1	0	—
FN–C3Hf	81	22.4	3	21.0
(B × C3H)F_1	85	27.0	3	30.0
FN–C3Hf	90	22.5	9	26.1
(BC3H × C3H)BC_1	86	24.0	5	24.6
FN–C3Hf	64	21.4	0	—
(BC3H × C3H)BC_2	87	23.0	6	24.7
FN–C3Hf	59	20.5	1	24.0
(BC3H × C3H)BC_3	56	19.5	2	22.0
FN–C3Hf	67	15.6	2	20.5
(BC3H × C3H)BC_4	64	16.3	0	—
FN–C3Hf	41	15.8	1	25.0
(BC3H × C3H)BC_5	38	14.8	0	—
FN–C3Hf	33	13.7	0	—
(BC3H × C3H)BC_6	34	12.8	1	28.0
FN–C3Hf	67	15.4	2	26.5
(BC3H × C3H)BC_7	91	13.9	1	26.0
C57BL	20	23.7	0	—
FN–C3Hf	90	24.3	5	24.2
(B × C3Hf)F_1	98	27.6	1	32.0
FN–C3Hf	77	22.3	3	23.7
(BC3Hf × C3Hf)BC_1	87	24.3	6	24.5
FN–C3Hf	79	22.7	4	26.0
(BC3Hf × C3Hf)BC_2	72	22.1	5	24.8
FN–C3Hf	61	19.2	1	23.0
(BC3Hf × C3Hf)BC_3	52	21.2	0	—
FN–C3Hf	78	18.8	1	23.0
(BC3Hf × C3Hf)BC_4	95	17.0	0	—
FN–C3Hf	63	17.2	0	—
(BC3Hf × C3Hf)BC_5	59	19.3	2	22.5
FN–C3Hf	39	19.8	2	23.0
(BC3Hf × C3Hf)BC_6	40	19.5	0	—
FN–C3Hf	57	20.4	2	25.0
(BC3Hf × C3Hf)BC_7	72	19.5	1	24.0

*See reference 5.

†FN—These and subsequent FN groups were strain C3Hf females (of the line without the mammary tumor agent) foster-nursed on the females of the preceding group to test them for the presence or absence of the agent.

‡B—C57BL.

Age at tumor development.—The spontaneous sarcomas occurred in mice of very advanced age (table 1). In 1 group a sarcoma appeared at 17 months (the only sarcoma in that group) but in all other groups in which these tumors occurred the average age was over 20 months, and in a few it was over 30 months.

It is evident that one of the reasons so many spontaneous sarcomas were found in the animals of this study was because many mice lived to advanced age. However, this does not account for the fact that none occurred in C57BL or C57BL backcross mice, for many of these animals also lived to comparable ages.

Coexistence with other neoplasms.—Forty-three fibrosarcomas, or 40.5 percent, were found in mice which had other neoplasms, but in only 3 cases were fibrosarcomas found at more than 1 site in the same mouse. The most frequent coexistent tumors were hepatomas, ovarian tumors, lung tumors, and reticular neoplasms. All these tumors are commonly found in old strain C3H mice without the mammary tumor agent, and the association with fibrosarcomas was not beyond the expected frequency. It is notable that only 1 mouse with a fibrosarcoma also had a mammary tumor. Early death from mammary tumors probably prevented the development of fibrosarcomas at a more advanced age in susceptible strain C3H mice with the agent.

Location.—Thirty-nine tumors were located in mammary-gland areas, but did not appear to be derived from the gland. Since mammary-gland tissue extends over such a large area in the subcutaneous tissues of female mice, it was to be expected that a large proportion of the tumors would be found in mammary-gland areas. Twenty tumors were described as infiltrating the body wall, and often the ribs also. Eleven tumors were found in the thigh. Unusual locations were the base of the ear, the margin of the anus, and the tail. However, the sites were so scattered that no one seemed to have a greater predilection for sarcoma than another.

Histology.—The 106 subcutaneous sarcomas were composed in large part or entirely of spindle cells arranged in interlacing bundles. Sections from the tumors were routinely stained with hematoxylin and eosin. A number of these sections representative of particular histologic features were later stained with toluidine blue, van Gieson, the Wilder method for reticulum, and the periodic acid-Schiff method. No phosphotungstic acid-hematoxylin or fat stains were done, since all the tissues had been fixed in Fekete's modification of Tellyesnizcky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part).

Seventy-six of the tumors were composed of long, slender fusiform cells, of a fairly uniform size, separated by varying amounts of intercellular material. The cells and fibers were arranged in interlacing bundles (figs. 1, 2, and 3). Areas in the sarcomas usually assumed 1 of 2 microscopic patterns, such as Stout (*14*) described for tumors in man. The cells and fibers formed bundles that curved gently (fig. 1) or the bundles of cells and fibers bent sharply at right angles when they met, so that the cells and fibers were parallel for only a short distance in a single plane of

sectioning (fig. 2). Reticulum stains distinguished these patterns sharply, for each of the tumor cells was outlined with reticulum fibers. The tumors seldom showed the same structure in all areas, and a variety of patterns was usually found in a single section. The amount of intercellular substance varied, as did the shape and size of the cells. A focus composed of larger and more oval cells might compress adjacent tumor tissue (fig. 3), suggesting a difference in growth rate in different areas of the tumor. Such structural variations in different areas in a single tumor were displayed clearly in the tumor removed from the pancreatic region (figs. 4 and 5). One area of this tumor was composed of large oval or round cells showing great variation in size. This was sharply separated from an area composed of infrequent small, oval cells with an abundance of intercellular collagenous material (fig. 4). Another area in the same tumor was composed of elongated cells with deeply stained nuclei, separated by bundles of dense collagenous tissue (fig. 5).

Some margins of the tumors were sharply outlined by a connective-tissue capsule which probably resulted from compression of adjacent tissues (fig. 6). Other margins, often in the same section, showed diffuse infiltration of muscle or fat by sarcoma. Frequently, a collagenous or edematous tissue containing very few cells was found adjacent to a characteristic cellular fibrosarcoma, separating it from the non-neoplastic tissue (fig. 7). Toluidine blue showed no metachromasia of surrounding tissue and no increase in the number of mast cells at the tumor margin. Scattered mast cells often appeared within the tumor, usually near the edges, and were probably pre-existing in the normal tissue. No close association between the mast cells and tumor cells, such as has been reported in fibrosarcomas of rats, was found (*15*).

Most of the tumors showed a few much larger cells among the uniform spindle-cell population (fig. 2). In 9 tumors, however, large, broad, straplike cells predominated in many areas (figs. 8 and 9) and suggested origin from muscle tissue. However, no cross striations could be found, and since fibroblasts are capable of assuming this morphologic form, it was concluded that these tumors probably were fibrosarcomas. Another difficulty in deciding whether some tumors originated from muscle cells was the infiltration of the normal muscle by the sarcomas. The tumor tissue could often be seen extending through the panniculus carnosus. All the muscle fibers disappeared in the central area, while, at the margins, the muscle bundles became widely separated by the infiltrating tumor tissue. The perimysium was a frequent route for extension (fig. 10), and often muscle fibers with multinucleate cells persisted in the midst of sarcoma cells (fig. 11).

Lymph nodes were included in several sections of the sarcomas. The neoplastic tissue, however, was not seen to infiltrate diffusely the lymphoid tissue as it did muscle, and often the node was completely surrounded but not invaded (fig. 12).

As already described, a number of tumors showed, at their margins, tissue composed of relatively few cells and an abundant intercellular

matrix. Mitotic figures were infrequent in this tissue. Twenty-one tumors were composed almost entirely of this type of tissue, with only a few islands of a more cellular structure (figs. 10, 12, 13, and 14). These lesions sometimes resembled an inflammatory fibrosis, but the absence of leukocytes or abscess formation made this diagnosis improbable. Moreover, the infiltrative capacity of the tissue and the accompanying destruction and replacement of normal cells left no doubt that these were progressive, destructive neoplasms. With the periodic acid-Schiff method, the poorly cellular areas showed great variation in the amount of Schiff-positive substance, and ranged from practically no coloration to an intense pink. Likewise, staining by the van Gieson method varied. In some tumors (fig. 13) the tissue was intensely stained for collagen, which appeared to be abundantly produced by the neoplastic cells. Reticulum fibers were generally few in the sparsely cellular areas. It was noted at autopsy that tumors of this histologic structure were often firm and difficult to cut. In those mice in which the ribs and intercostal muscles were extensively infiltrated, the marrow tissue of the ribs was often largely replaced by tumor (fig. 14).

Discussion

Enough information was given in the report by Slye, Holmes, and Wells on subcutaneous sarcomas in the noninbred Slye mice to warrant a comparison with the present observations on similar tumors in inbred strains of mice and their F_1 and backcross hybrids. The incidence of fibrosarcomas in the mice of the present study was 106 in 4,049, or 2.6 percent, considerably above the 0.7 percent of spindle-cell sarcomas reported in the noninbred group. In the C3H, C3Hf, and C3H backcross groups combined the incidence was 3.1 percent. However, all the inbred, hybrid, and backcross mice of the present study were females, and the majority were of advanced age. No information on age was given in the early report of Slye, Holmes, and Wells, and both sexes were included. They found the tumors were twice as frequent in females as in males. About half the tumors in the Slye mice were located in the mammary-gland area, and about half of these were thought to arise from mammary-gland tissue. In some instances the relation to the ducts was so close as to resemble a "pericanalicular fibrosarcoma." Only 5 tumors among the inbred, F_1, and backcross hybrid mice of the present study appeared to be derived from the mammary-gland tissue. Osseous and periosteal tissues were the sites of origin in 11 mice in the Slye report and in 8 in the present series. Other differences were 21 sarcomas in internal organs against 6 in the present report and only 3 in the abdominal and chest wall compared to 20 at similar locations in the present series. Multiple sarcomas were found in 5 mice of the Slye stock and in 3 mice in our series. Tumors other than sarcomas coexisted in 23 percent of the noninbred Slye mice, and in 40.5 percent of our mice. Pulmonary tumors were the most frequent of the coexistent tumors in the Slye mice and mammary-gland carcinomas were also found in 4 mice which had

sarcomas. Only 1 mouse in our series had both a mammary tumor and a sarcoma. Metastases were found in 13 percent of the spindle-cell sarcomas in the Slye mice and in none of the present series. One reason for this difference may be that mice of the present series were usually killed soon after the tumors became palpable.

Regarding the histology of the tumors in the Slye, Holmes, and Wells report, it was stated that "spindle cell sarcomas" were the most abundant, constituting over half of the tumors, but all possible histologic types were illustrated—from the fibrosarcomatous or "desmoid" [3] growths to very large-celled sarcomas with uninuclear and multinuclear giant cells. It was considered unnecessary to describe the characteristics of the spindle-cell sarcomas in detail, for they corresponded in structure "absolutely with the sarcomas occurring in man and given the same designations." In the present series, likewise, the majority of the tumors closely resembled fibrosarcomas found in man. The early report, however, did not describe any tumors with few cells and the abundant intercellular substance such as appeared in our series. It may be that our practice of killing the mice as soon as the tumors became palpable is the reason that the less cellular tumors were found so frequently. If the tumors has been allowed to reach a larger size, the more cellular areas might have overgrown and replaced the less cellular tissue, assuming that the more cellular tissue had a more vigorous growth rate.

Slye, Holmes, and Wells seriously considered that trauma might have caused some of the tumors in their mice, and noted that in 11 cases the tumors arose at the site of trauma. No association with trauma was noted in the present series.

If the spontaneous subcutaneous sarcomas are compared with tumors induced by the injection of carcinogens, many similarities are found. However, most of the spontaneous tumors suggest a fibroblastic origin, whereas many induced tumors are thought to originate in smooth or striated muscle. As Stewart (*1*) pointed out, on subcutaneous injection of a carcinogen a variety of different types of cells may be directly exposed to the carcinogen, and the classification of the resulting tumors becomes extremely difficult. Saxén (*16*) noted that C3H mice exposed at 2 or 3 weeks of age to methylcholanthrene developed many sarcomas of muscle-cell origin, while those exposed at 3 months or a year developed fibrosarcomas. Descriptions of induced neoplasms have not included tumors with much collagenous intercellular substance and relatively few cells. The carcinogen-induced tumors were probably more rapid in their development and anaplastic cells more frequent.

Many of the tumors resembled those developed by Earle in tissue culture. None of the spontaneous tumors, however, showed the extreme alteration of Earle's strain O, which had the longest exposure to methylcholanthrene, but they frequently resembled the control strains with no exposure, and strain H with the shortest exposure (*17*).

Histologically, the tumors showed the variations in different areas

[3] The photograph of a "desmoid" growth closely resembles our figure 1.

often observed in human fibrosarcomas. The tumors, therefore, were probably not composed of a uniform cell population, all with the same growth potential. In this respect they differed from most transplanted sarcomas of the mouse in which a selection for the more vigorously growing cells probably occurs. Tumors composed largely of a poorly cellular tissue with abundant collagen production, though often resembling granulation tissue or areas of fibrosis, displayed remarkable infiltrative capacity, and were as truly neoplastic as the more highly cellular tumors with many mitoses. The neoplastic cell in these tumors probably represented an aberrant functional response in the fibroblast, which led to an overproduction of collagen. The general statement that a neoplastic tissue is hypofunctional and undifferentiated has many exceptions. Overactivity of a particular function is not uncommon in certain tumors in mice, for example, hyperkeratosis in squamous-cell carcinomas of the forestomach, the secretory activity in some cells of mammary tumors, excessive production of glycogen by some hepatoma cells, and the phagocytic activity in some reticulum-cell sarcomas.

A tissue with few cells and much fibrous tissue at the margin of many of the fibrosarcomas may represent a remnant of less malignant tissue, most of which had been overgrown and obscured by foci having greater proliferative capacity. The few tumors that were composed entirely of less cellular tissue may correspond to so-called benign fibromas in man. The growth rate of these sarcomas in the mouse is not known and no attempt was made to transplant them.

The histologic pattern of the tumors was generally similar to that of the sarcomas developing in C3H mice and their hybrids, which received injections of various filtrates when newborn. In the mice injected when newborn, however, the sarcomas appeared in a younger age group, they were often multiple and associated with parotid-gland tumors (*7*).

Summary

One hundred and six sarcomas, probably derived from fibroblasts of the subcutaneous tissue, were found in a study of 4,049 untreated female mice of strain C3H with the mammary tumor agent, strain C3Hf without the agent, strain C57BL, and their F_1 and backcross hybrids. The sarcomas occurred in the C3H, C3Hf, F_1, and C3H and C3Hf backcross mice, with none occurring in the C57BL or C57BL backcross females.

Histologically, the majority of the tumors resembled fibrosarcomas induced in mice with carcinogens and the fibrosarcomas developing in man. One type is described in which cells were few in number and fibrous tissue was abundant. It has not been described in mice exposed to carcinogens, and is not included among malignant fibrosarcomas in man.

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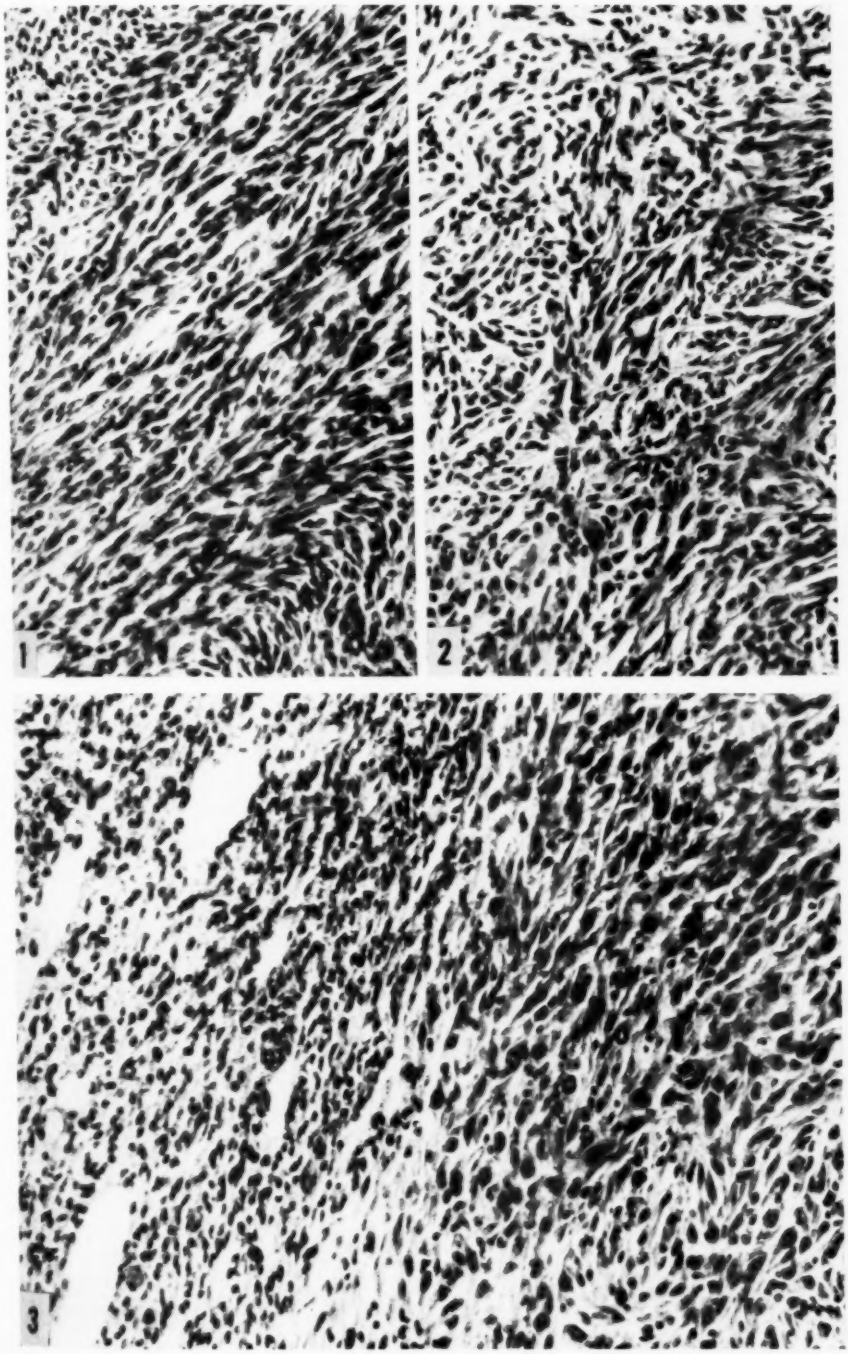
PLATE 49

All sections in photographs stained with hematoxylin and eosin

FIGURE 1.—A frequent pattern of fusiform cells in a fascicular arrangement, with interlacing bands of cells extending for a considerable distance in one plane. Long axes of the cells are parallel. Each of the cells is invested by delicate reticulum and collagen fibers. Many mitotic figures. × 250

FIGURE 2.—Another frequent pattern. Bands of cells extend only short distances in one plane. Cells generally of uniform size, but one much larger cell in *lower left*. × 250

FIGURE 3.—Line of separation between 2 areas in a sarcoma. Tissue on *left* composed of smaller cells with much intercellular material and a fascicular arrangement. Nodule on *right* is composed of large oval cells with abundant cytoplasm and little intercellular substance. It compresses tissue on *left*, producing a band of flattened, crowded cells immediately adjacent to it. × 250



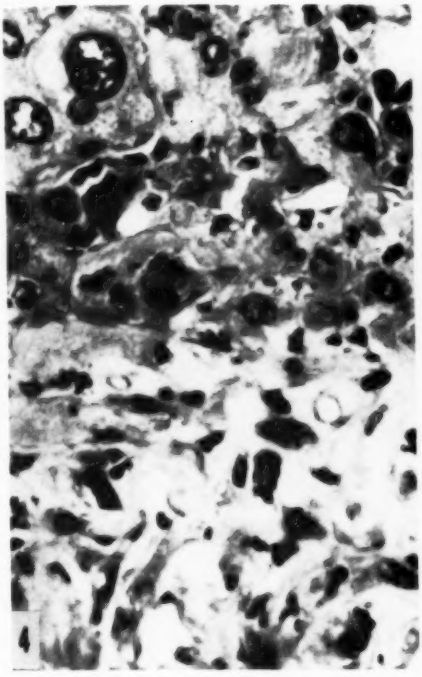
395335—56——15

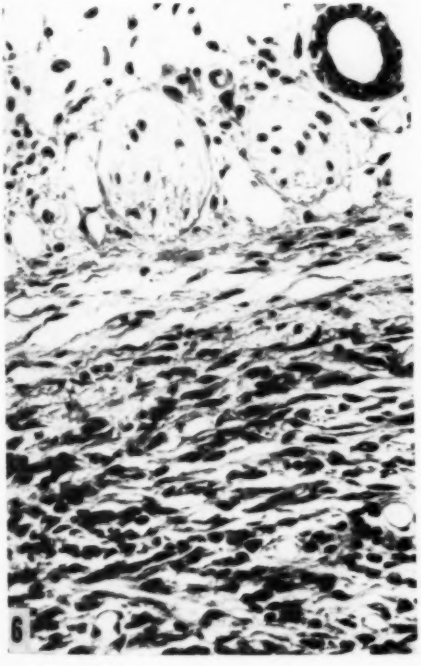
PLATE 50

FIGURES 4 and 5.—Variation in morphology in different areas of a sarcoma found in the region of the pancreas. 4) Tissue at *top* contains cells of different size, some with very large nuclei and abundant cytoplasm. Tissue at *bottom* is composed of relatively few cells, with abundant intercellular material. × 460 5) Cells from another area are elongated and the nuclei appear shrunken. Abundant collagen formation between cells. × 460

FIGURE 6.—Margin of a sarcoma. Adjacent fibrous tissue is compressed, forming a capsule. Cross sections of nerve fibers and a mammary duct are shown above. × 250

FIGURE 7.—A cellular fibrosarcoma on *right* adjoining an area containing few cells and abundant, loose fibrous tissue. Non-neoplastic connective tissue appeared farther to the *left*. × 350





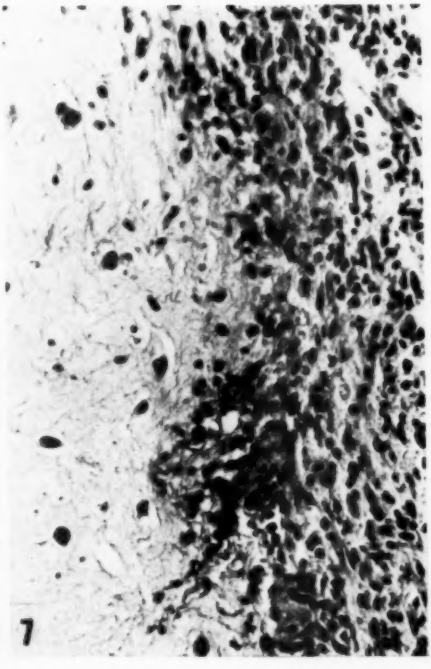


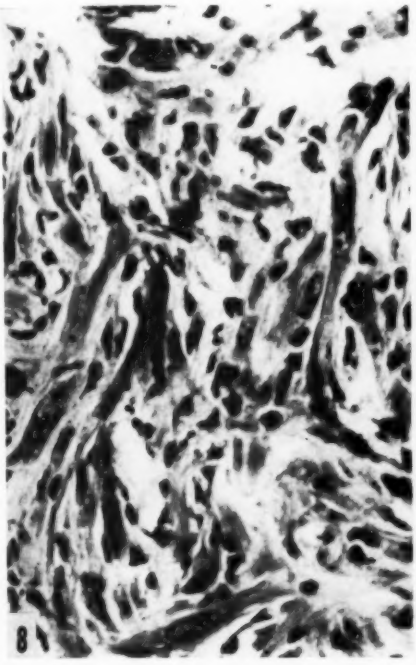
PLATE 51

FIGURE 8.—Area in a sarcoma with many long, straplike cells with very large nuclei. No cross striations could be seen. × 250

FIGURE 9.—Sarcoma containing many giant cell forms. Cells in this tumor were generally oval and plump, and suggested muscle-tissue origin. Collagen was scanty. × 250

FIGURE 10.—Infiltration of individual striated muscle fibers by fibrosarcoma. Fibers are widely separated by tumor cells, forming abundant collagenous matrix. × 250

FIGURE 11.—A muscle fiber with multiple nuclei surrounded by infiltrating fibrosarcoma. Multiplication of muscle-cell nuclei is frequent in skeletal-muscle cells entrapped in the midst of infiltrating sarcomas. × 250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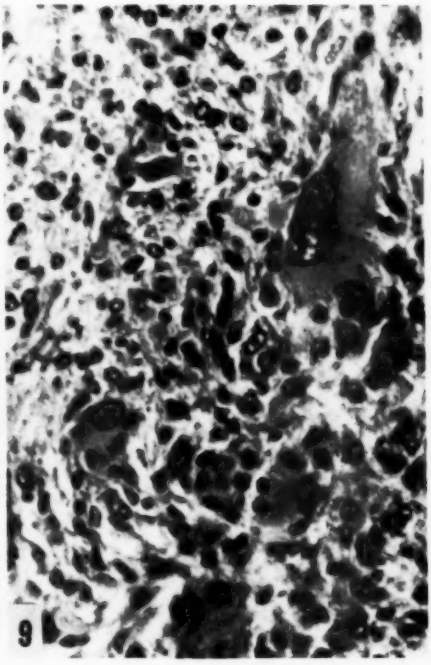
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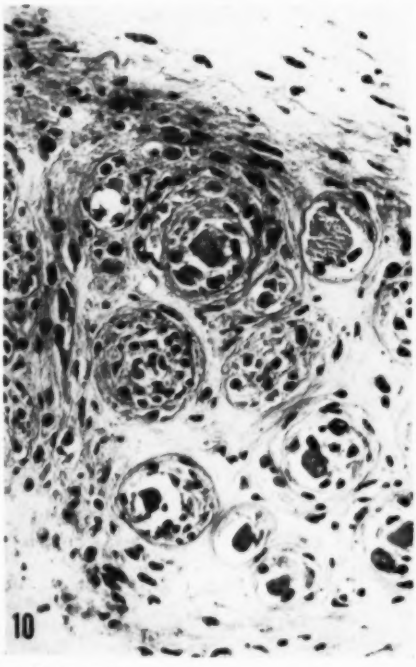
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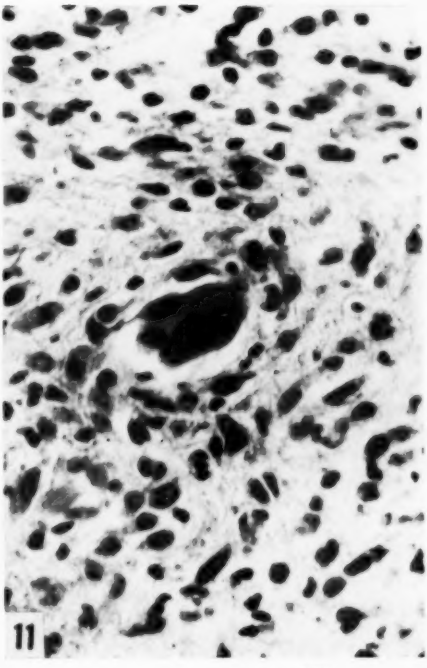
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PLATE 52

FIGURE 12.—Relatively acellular collagenous fibrosarcoma that has infiltrated the muscle diffusely but has stopped sharply at the margin or capsule of a cystic lymph node. The sarcomatous tissue was heavily stained by the van Gieson method. × 33

FIGURE 13.—Small cellular tumor mass on *right* surrounded by relatively acellular collagenous tissue in which mammary ducts are embedded. × 60

FIGURE 14.—Cross section of a rib and adjacent intercostal muscle. Neoplastic tissue containing few cells but much collagen has infiltrated between the muscle bundles and replaced part of the marrow. × 60

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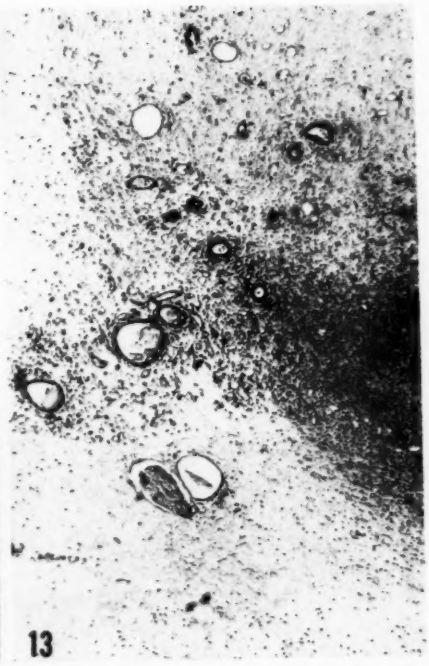
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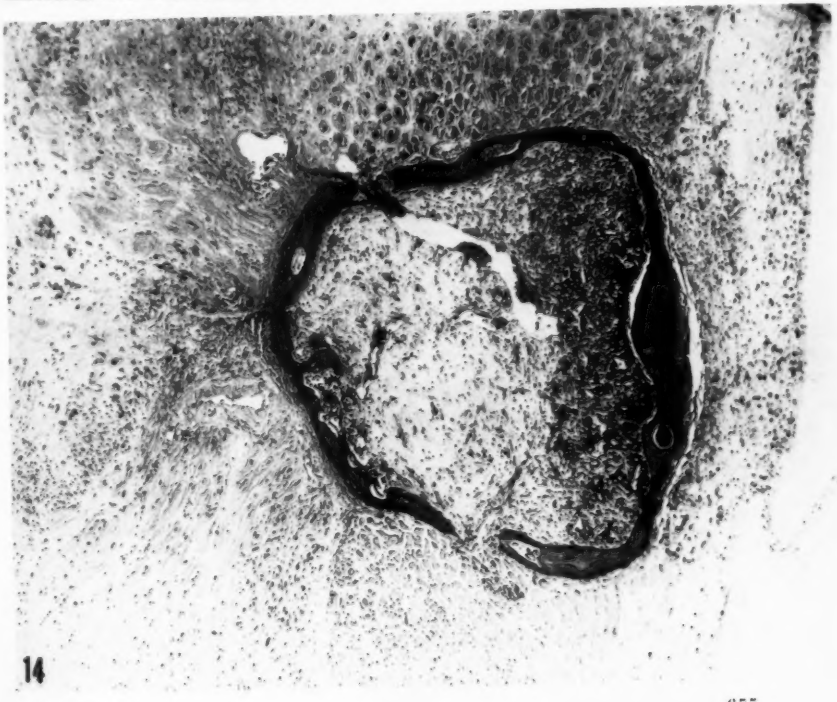
14

Effect of *in Vitro* X Irradiation on Pigmented and Pale Slices of Cloudman S91 Mouse Melanoma as Measured by Subsequent Proliferation *in Vivo*[1, 2, 3, 4]

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Human malignant melanomas are generally considered to be resistant to radium and X-ray therapy (*1,2*). The Cloudman S91 malignant mouse melanoma showed no inhibition of growth following injection of the radiomimetic chemical bis(β-chloroethyl)-sulfide (mustard gas), whereas certain mouse lymphomas were damaged (*3*). The Harding-Passey mouse melanoma was resistant to 2 other such agents, namely, 2,4,6-tris(ethylenimino)-*s*-triazine (TEM) and methoxypyridoxyl-bis(β-chloroethyl)-amine, while various other test tumors were sensitive to the drugs (*4*). However, the related compound triethylene thiophosphoramide had a marked inhibitory effect on this tumor (*5*). This melanoma was found to be extremely resistant to rays emitted from embedded radon seeds (*6*).

Since human experimentation was not practicable, we have attempted to elucidate the phenomenon of melanoma resistance to radiation in animals, using the Cloudman S91 melanoma. A detailed presentation of the methods and results of our approach follows, but, briefly, our procedure was to expose, *in vitro*, tissue slices of pigmented and pale areas of the same tumor to varying doses of X rays and then to implant them immediately in untreated strain DBA mice, the strain of tumor origin.

Materials and Methods

The Cloudman S91 melanoma originated in a DBA/Jax female mouse and was first described in 1937 by Dr. Cloudman (*7*), of the Roscoe B. Jackson Memorial Laboratory. It has been carried in the laboratory of origin and at the National Cancer Institute. Experiments described in this paper were performed with melanomas derived from the S91-DBA-II

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[2] Presented in part at the meeting of the American Association for Cancer Research at San Francisco, April, 1955.

[3] Acknowledgment is made of the indispensable technical assistance of Mable Amano and Dorothy Walker.

[4] This investigation was supported in part by research grants C-1986 and C-2500 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and by a grant from the Damon Runyon Memorial Fund for Cancer Research.

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395335—56——16

line, sent to us through the kindness of Dr. Glenn Algire. The biology and histopathology of the pigmented and nonpigmented tumors, carried in strain DBA and strain C mice, respectively, have been described by Algire (*8*) and Loustalot, Algire, Legallais, and Anderson (*9*).

The tumor was received in our laboratory in the 67th transfer generation and has been perpetuated by subcutaneous transplantation in male and female pen-bred DBA mice from the colony of Dr. Arthur Kirschbaum. The host mice used for routine transfers and experiments weighed between 25 and 30 gm.

Experiments cited here were designed to determine the effect of X rays on pigmented and pale slices from the same melanoma specimen. The effects were evaluated from the following information:

(1) Length of time before palpable tumor was found.
(2) Percentage of animals with growing tumor implants.
(3) Rate of tumor growth.
(4) Life span of the hosts.
(5) Histologic differences.
(6) Percentage of cells with melanin in pigmented and pale slices prior to irradiation and implantation.
(7) Mitotic-index values in the same tissues as (6) for cells containing melanin.

For items 1 through 5, data were applied to the *in vivo* experiments—for pigmented and pale control and irradiated tumor implants in host mice. Criteria 6 and 7 were applied to a series of pigmented and pale tissue slices prior to implantation.

There were 4 groups of melanoma tissue slices prepared in each experiment, as follows: group 1, pigmented tumor slices, untreated (controls); group 2, pale tumor slices, untreated (controls); group 3, pigmented tumor slices, irradiated; group 4, pale tumor slices, irradiated.

Four experiments were undertaken. In the first 3, the growth in host mice of both pigmented and pale melanoma implants was observed after *in vitro* irradiation with 2500, 2800, and 3000 r, respectively. In the 4th experiment, only pigmented melanoma implants were used, and the radiation dose was 3500 r.[6]

Then, cytologic analyses of untreated pigmented and pale tumor slices similar to those used for irradiation and transplantation experiments were performed.

Tissue slices were prepared aseptically and weighed (approximately 10 mg. wet weight). They were then irradiated in covered petri dishes in 1 time span. Tissues were exposed for varying lengths of time, depending on the total amount of radiation desired.[7] Immediately after irradiation, treated and control tissue slices were implanted by subcutaneous injection with a 13-gauge trocar to the right axillary region of male and female mice.

[6] Maximow double-coverslip cultures were set up, using standard tissue-culture techniques. The substrate consisted of a plasma-embryo extract clot. The supernatant feeding solution was a mixture of human cord serum, chick-embryo extract, and Tyrode's balanced salt solution.

[7] Physical factors were as follows: 64 KVP, 5 Ma., 0.25 mm. Cu and 1.00 mm. Al added filter, 12. 5 cm. tissue-target distance, 96 r per minute (after calculating 5% loss through petri-dish top).

Mice were placed in 1 of the 4 groups according to the type of injected implant received. There were usually 12 mice in each group.

Diameters of growing implants were measured weekly by means of vernier calipers. Animals bearing growing tumor were generally allowed to die but occasionally a mouse was sacrificed. Tumors were fixed in 10 percent neutral formalin and paraffin sections 6μ thick were then stained with hematoxylin and eosin. For the cytologic analyses untreated tissue slices were fixed immediately following excision and prepared by the same histologic procedures.

Slides for the cytologic analyses were examined through the microscope to determine the number of tumor cells containing melanin (endogenous and exogenous melanin) and macrophages (exogenous melanin). One thousand cells were counted in each tissue slice. The identical sections used for melanin counts were also examined for the proportion of dividing cells to resting cells (mitotic index) using Goldfeder's (*10*) modification of Chalkley's quantitative procedure (*11*). This procedure involved the use of a specially designed reticule with 5 pointers inserted in the eyepiece. Chalkley demonstrated that stabilizing ratios of various tissue components could be obtained from 500 "hits," or counts, of intact cells. Goldfeder extended the number of hits to 700.

Counts of melanized cells, together with mitotic-population counts on identical cells, served to clarify differences between pigmented and pale tumor tissue used for the irradiation and implantation experiments.

Results

Experiments 1, 2, 3, and 4

The experimental data, summarized in table 1, show the results of *in vitro* irradiation of melanoma tissue on subsequent transplantability ("take") and growth in host mice. The percentage of successful takes of control and irradiated tissue following implantation in host mice varied in relation to the irradiation dose and to the use of pigmented or pale areas (table 1). An increase in X-ray dose was accompanied by a decrease in the number of successful takes in host mice. Nonirradiated (control) pigmented implants in experiments 1 through 4 grew in 91 to 100 percent of host mice. Control pale implants grew in 70 to 83 percent of host mice.

Following irradiation at 2500, 2800, and 3000 r, tumors developed in 83, 91, and 66 percent, respectively, of the hosts bearing pigmented implants, in contrast to 50, 16, and 0 percent in hosts bearing pale tumors.

As table 1 also shows, the length of time before tumor could be observed was 3 weeks in hosts bearing untreated (control) pigmented or pale tumor. This period was increased by 3 weeks in experiment 1, in which implants were first irradiated with 2500 r. The time before palpable tumor of all growing implants, following exposure to 2800 r, was 1 week longer than that for controls. There was no increase in this period for surviving implants after irradiation with 3000 r.

Having established the *in vitro* X-ray dose at which successful growth of

TABLE 1.—*Results of implantation of control and irradiated pigmented and pale melanoma tissue in DBA mice*

Experiment No.	Tissue type	Number of mice	Dose (*r*)	Takes (*percent*)	Time before palpable tumor (*weeks*)	Life span of host mice (*weeks*)
1	Pigmented	12	0	100	3	9–13
	Pale	12	0	83	3	10–13
	Pigmented	12	2500	83	6	14–17
	Pale	12	2500	50	6	14–17
2	Pigmented	12	0	91	3	10–14
	Pale	12	0	79	3	10–14
	Pigmented	12	2800	91	4	11–15
	Pale	12	2800	16	4	11–15
3*	Pigmented	24	0	95	3	10–12
	Pale	24	0	70	3	10–14
	Pigmented	24	3000	66	3	12–16
	Pale	24	3000	0	—	—
4	Pigmented	8	0	100	3	11–15
	Pigmented	8	3500	0	—	—

*This experiment was repeated, using a new series of mice, with identical results.

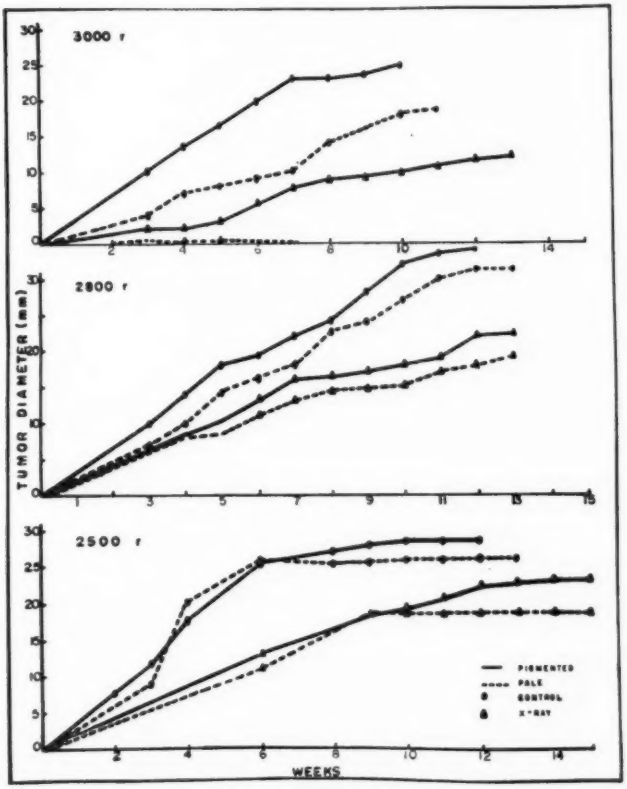
pale tissue was prevented in the hosts, the dose was then increased to 3500 r in experiment 4. At this dose, none of the mice bearing irradiated pigmented tissue developed tumors, while all receiving untreated (control) melanoma tissue developed tumors.

Slices of pigmented control and irradiated tumor of experiment 4 were prepared for tissue culture. Both control and irradiated tissue produced cellular outgrowths in culture. Typical bipolar melanoma cells, melanocytes, and macrophages were observed. The X-ray dose of 3500 r may be regarded as the attenuating dose [as defined by Goldfeder (*12*)] for pigmented melanoma tissue.[8]

Data recorded in table 2 and text-figure 1 indicate the mean values of tumor diameters in mice bearing control and irradiated implants. The tumor diameters of pale implants in all experiments were less than those of pigmented implants. Irradiated pigmented and pale implants which survived treatment grew steadily in host mice (table 2) and were consistently smaller than nonirradiated pigmented and pale implants. The increase in life span of mice bearing tumors previously irradiated at 2500 and 3000 r appeared to be related to an initial slower rate of tumor growth.

Control pigmented and pale implants that developed in host mice were histologically similar to the usual type of transplantable melanoma tumors, which have been described in detail by Loustalot, Algire, Legallais, and Anderson (*9*). They were solidly cellular with little stroma. Cells were polymorphous and areas varied in appearance from carcinoma-like to sarcoma-like. Individual melanoma cells had vesicular nuclei and

[8] The attenuating dose is the dose of irradiation which, when administered to tumor fragments *in vitro*, prevents their growth *in vivo* (after transplantation into the mice) but does not prevent their proliferation *in vitro* (tissue culture).



TEXT-FIGURE 1.—The effect of *in vitro* X irradiation of pigmented and pale melanoma implants on growth in host DBA mice following transplantation. This chart is a visual representation of table 2.

cytoplasm often filled with diffuse yellow-brown melanin and occasional melanin clumps. Macrophages, often packed with engorged melanin, were dispersed among melanoma cells.

All tumors growing from irradiated pigmented implants had central necrosis. Surrounding the necrotic core was an intermediate zone of pyknotic cells and plasma cells. The outer zone was composed of uninjured tumor cells identical with those in nonirradiated tumors. Cells in various stages of mitosis were observed. There were many more macrophages filled with melanin. Papillary proliferation of melanoma cells in the lumen of blood vessels was frequent. Often small patches of necrotic tissue were present in peripheral areas completely walled off by uninjured tumor cells.

The tumors from irradiated pale implants in experiments 1, 2, and 3 were light tan to dark gray macroscopically. Microscopic observation showed a large central necrotic region in which islets of uninjured, nonpigmented tumor cells were found. There were many large, binucleate cells scattered

TABLE 2.—*Growth* in vivo *of control and irradiated pigmented and pale melanoma implants**

Weeks	Irradiation with 2500 r				Irradiation with 2800 r				Irradiation with 3000 r†			Irradiation with 3500 r‡
	Pigmented control	Pale control	Pigmented irradiated	Pale irradiated	Pigmented control	Pale control	Pigmented irradiated	Pale irradiated	Pigmented control	Pale control	Pigmented irradiated	Pigmented control
2												
3	8.0±2.6 (12)	11.0±2.5 (10)			10.0±1.2 (11)	7.0±1.7 (9)			10.4±1.8 (23)	3.2±1.0 (17)		5.2±0.8
4	17.2±2.4 (12)	20.0±2.9 (10)			14.0±1.8 (11)	10.0±1.9 (9)	8.0±1.7 (11)	8.0±0.8 (2)	13.3±2.4 (22)	7.2±1.5 (17)		7.3±1.8 (8)
6	25.6±2.8 (12)	25.8±3.0 (10)	13.0±2.6 (10)	11.0±2.4 (6)	19.4±3.1 (11)	14.7±2.3 (9)	13.0±1.9 (11)	11.0±0.9 (2)	21.4±2.7 (22)	9.0±2.0 (17)	5.0±1.5 (16)	12.4±2.1
8	27.1±3.3 (12)	25.4±3.0 (10)	15.0±2.2 (10)	14.0±3.0 (6)	24.0±3.2 (11)	22.8±2.5 (9)	16.4±2.1 (11)	14.2±1.2 (2)	23.0±3.0 (22)	14.0±2.8 (17)	9.0±1.7 (16)	20.0±3.0
9	28.0±3.5 (10)	25.0±3.0 (10)	18.3±2.0 (10)	18.5±1.9 (6)	28.0±2.9 (11)	22.4±3.0 (9)	17.0±2.2 (11)	14.8±1.5 (2)	23.5±3.1 (20)	16.2±3.0 (17)	9.2±1.5 (16)	24.0±2.2
10	28.0±3.8 (7)	25.0±2.8 (10)	19.0±2.0 (10)	18.2±2.7 (6)	32.0±2.4 (11)	26.8±3.0 (7)	18.2±2.0 (9)	15.0±1.8 (2)	30.1±2.7 (11)	10.0±2.7 (12)	10.0±1.8 (12)	26.5±1.9
11	28.2±3.8 (5)	25.0±3.5 (7)	21.0±2.2 (10)	18.4±2.9 (6)	35.0±2.9 (8)	30.4±2.0 (7)	19.0±1.9 (6)	17.0±1.8 (2)	30.0±2.7 (8)	11.2±3.0 (7)	11.5±2.1 (7)	28.8±1.7
12	28.0±2.9 (2)	25.0±3.0 (3)	23.0±2.3 (8)	18.2±3.2 (5)	36.0±2.6 (2)	31.2±4.0 (4)	22.0±2.2 (3)	17.9±1.2 (2)	30.0±2.0 (3)	11.5±2.0 (2)	12.0±1.7 (4)	
13			23.0±3.0 (5)	18.1±3.0 (5)		31.0±3.0 (2)	23.0±1.8 (2)	18.1 (1)			12.3 (1)	
14			23.0±2.0 (4)	18.1±2.8 (3)								
15			23.0 (1)	18.0 (1)								

*Each value represents average tumor diameter in millimeters ± S.D. Number in parentheses indicates number of tumors measured.

† No growth of pale irradiated implants was observed at 3000 r.

‡ No growth of pigmented irradiated implants was observed at 3500 r.

throughout healthy areas, which were similar to those described in irradiated tissue by other workers.

Tumors grown from both pigmented and pale irradiated implants were prepared for frozen section and stained for DOPA (dihydroxyphenylalanine) oxidase, using Laidlaw's modification of Bloch's test as described by Glick (*13*). DOPA-positive cells were present in both types of tissue, but there were fewer in the growing pale implants.

Cytologic Analyses

Differences in radiosensitivity of pigmented and pale tissue, as revealed in experiments 1 through 4, led to an analysis of an additional series of nonirradiated pigmented and pale tissue slices, similar to those used for irradiation and implantation.

Preliminary counts of stained sections of melanized cells in pigmented and pale areas of a few melanoma specimens were reported briefly (*14*). It was found that in pigmented areas 1 out of 5 cells contained visible melanin and in pale areas 1 out of 66 cells had melanin. Counts included cells with finely dispersed endogenous melanin and cells with aggregated exogenous melanin. Mitotic-index values for the same areas showed that in pigmented areas 1 of every 216 cells was dividing and in pale tissue 1 of every 361 cells.

To secure a better statistical evaluation of the 2 tissue types, a more extensive series of counts of cells with melanin and of mitotic indices seemed warranted. The reasons for differences in the radiosensitivity of pigmented and pale tissue might then become apparent. The data on this series are given in table 3. As seen in samples from 8 tumor specimens, pigmented areas consistently had a greater number of melanized cells per volume of tissue than did pale areas. However, there was a lack of consistency in mitotic-index values for pigmented and pale tissues. As seen in table 3, in 50 percent of the specimens (1, 4, 6, and 8) the mitotic index was higher in pigmented areas. Such a percentage value lies within the possibility of pure chance. Therefore, the higher mitotic index could not be regarded as a consistent property of pigmented areas.

Discussion

A larger number of pigmented than pale melanoma areas remained viable following irradiation at 2500 and 2800 r. No pale slices survived irradiation at 3000 r, while 66 percent of pigmented slices survived. A dose of 3500 r *in vitro* was sufficient to prevent growth of implants of pigmented tissue *in vivo* but did not alter its growth capacity in tissue culture.

Pale melanoma slices were relatively radiosensitive, compared to pigmented slices. Pigmented and pale areas were both sensitive to X rays *in vitro* when cell damage caused by *in vitro* irradiation at 3000 r is compared to that reported by Goldfeder (*10*) in the DBAH and C3HB transplantable mouse mammary tumors.

When pigmented and pale tumor cells survived irradiation, viable cells continued to divide, forming new tissue with no morphologic alterations.

TABLE 3.—*Number of melanized cells and mitotic-index values in histologic sections of untreated pigmented and pale mouse melanoma slices*

Tumor specimen	Tissue type	Ratio of melanized to nonmelanized cells	Melanized cells (*percent*)	Mitotic index	Mitoses (*percent*)
1	Pigmented	1:4	25.0	1:14	7.0
	Pale	1:27	3.7	1:22	4.5
2	Pigmented	1:.04	61.0	1:48	2.0
	Pale	1:25	4.0	1:40	2.5
3	Pigmented	1:6	17.0	1:55	1.8
	Pale	1:16	6.0	1:18	5.5
4	Pigmented	1:2	51.3	1:28	3.5
	Pale	1:7	13.9	1:63	1.5
5	Pigmented	1:5	20.0	1:25	4.0
	Pale	1:30	3.3	1:16	5.5
6	Pigmented	1:2.5	40.0	1:33	3.0
	Pale	1:8	13.0	1:37	2.7
7	Pigmented	1:2	50.0	1:230	0.4
	Pale	1:22	6.7	1:33	3.0
8	Pigmented	1:2	50.0	1:68	1.4
	Pale	1:4	25.0	1:78	1.2

Melanocytes maintained their ability to produce melanin as indicated by the positive DOPA reaction and the presence of visible melanin in surviving and growing implants.

The difference in radiosensitivity of pigmented and pale tissue is apparently related to the amount of melanin per unit volume of tissue and not to the number of mitotic stages. The relationship between radioresistance and melanin content as revealed in these experiments might best be explained by reviewing the comments of Commoner, Townsend, and Pake (*15*). Metabolically active tissues (*e.g.*, melanomas) possess a large number of free radicals which are normally associated with protein moieties and which are important in the action of visible (and probably also ionizing) radiation on biological systems. However, melanin-containing tissue has, in addition to free radicals, which are relatively instable, certain extraordinarily stable forms brought about by trapping and stabilization, within the polymer lattice, of unpaired electrons generated during reactions that lead to polymeric condensed ring structures (melanin). Such a stabilized system could account for the relative radioresistance of metabolically active pigmented melanoma tissue.

Summary

1) The *in vitro* irradiation of pigmented and pale slices of the Cloudman S91 mouse melanoma with 2500, 2800, or 3000 r revealed that a larger number of pigmented slices remained viable following treatment. Via-

bility was measured by the number of irradiated implants growing successfully in DBA mice, the strain of tumor origin.

2) A dose of 3000 r was sufficient to prevent subsequent growth *in vivo* of pale melanoma tissue slices. A dose of 3500 r was sufficient to prevent subsequent growth *in vivo* of pigmented slices but did not prevent growth in tissue culture. This dose may be regarded as the attenuating dose for pigmented melanoma slices.

3) Pigmented and pale melanoma slices which survived irradiation and grew in host mice did not lose their capacity to undergo mitosis and to form new melanin. These tumors were similar, histologically, to control tumors.

4) Counts of cells containing melanin and mitotic-index values for a series of pigmented and pale areas of 8 tumor specimens were evaluated. In pigmented tissue there were consistently more melanized cells per volume of tissue than in pale tissue. Mitotic-index values, on the other hand, followed no consistent pattern in relation to pigmentation areas.

5) The relatively greater radioresistance *in vitro* of pigmented, as compared to pale, tissue was accompanied by an increased number of melanized cells. An explanation for this phenomenon is discussed.

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Recorded and Expected Mortality among the Chinese of Hawaii and the United States with Special Reference to Cancer [1]

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A number of investigators have published analyses of clinical and autopsy material which support the idea that Chinese are subject to risks of cancer of several sites that differ from those observed in the white race. Some of these papers have demonstrated relative excesses for cancer of the liver (*1–3*) and pharynx (*4–7*) and a relative deficit of cancer of the prostate (*8–10*). Thus, this study is intended to expand previous observations by comparing mortality recorded among Chinese residing in the United States and Hawaii with deaths expected among them if they were subject to the same age- and sex-specific death rates experienced by the white and nonwhite populations of the United States. It is less likely that the differences in mortality thus demonstrated are due to different medical death-certification practices than when similar comparisons are made between Chinese dying in Asia and Americans dying in the United States.

Method of Study

Special tabulations of deaths attributed to malignancy during 1949 to 1952, inclusive, among Chinese residents in the United States and Hawaii were obtained from the National Office of Vital Statistics. These were compared to deaths expected among them during the same period if they had experienced the age-, sex-, and site-specific death rates of the United States whites or nonwhites for 1950 and 1951 (*11*). The ratio of observed deaths to expected deaths and the ratio of the Chinese average annual rate, adjusted for age by the indirect method, to the United States rate are the same. This ratio is referred to as the standardized mortality ratio (SMR).

The degree of reliance that one can place on differences demonstrated by these methods is to some extent dependent on the forces of mortality which cancer replaces, or those causes of death which may replace cancer. For this reason, Chinese mortality for all causes of death and for conventional broad causes has been compared to that of whites and nonwhites.

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The SMR's that were computed, however, refer only to the Chinese of the United States because recorded deaths, other than for cancer, were not readily available for Chinese in Hawaii.

General Results

In table 1 are tabulated the observed deaths for all causes and for broad causes occurring in the Chinese of the United States during 1949 to 1952. Also shown in this table and in text-figure 1 are the deaths expected to occur among them during the same period had they had the same mortality as the white or nonwhite populations of the United States recorded in 1950 and 1951. In addition, the resultant SMR's are presented. It is readily observed that the Chinese males of the United States have 30 percent higher total mortality than white males and that the majority of the broad causes contribute significantly to this excess. The remainder, including diseases of the circulatory system, do not differ significantly from the white race. When compared to total mortality recorded for nonwhite males, the adjusted Chinese male rate is lower. Mortality for a few of the broad causes among Chinese males is significantly higher than that of the nonwhite males, while mortality assigned to most of the broad causes is significantly lower. The adjusted Chinese female mortality is also significantly lower than that of the nonwhite females for all causes and for most of the broad causes of death. Chinese female mortality for only 1 broad cause is higher than that of nonwhite females, namely, deaths due to congenital malformations. Less difference is observed between the adjusted Chinese female mortality and that of white females than in any of the other 3 comparisons. A significant observation is the fact that mortality assigned to senility and ill-defined causes is comparable to that recorded for white males and females and much less than that of nonwhites of either sex.

Mortality Attributed to Cancer

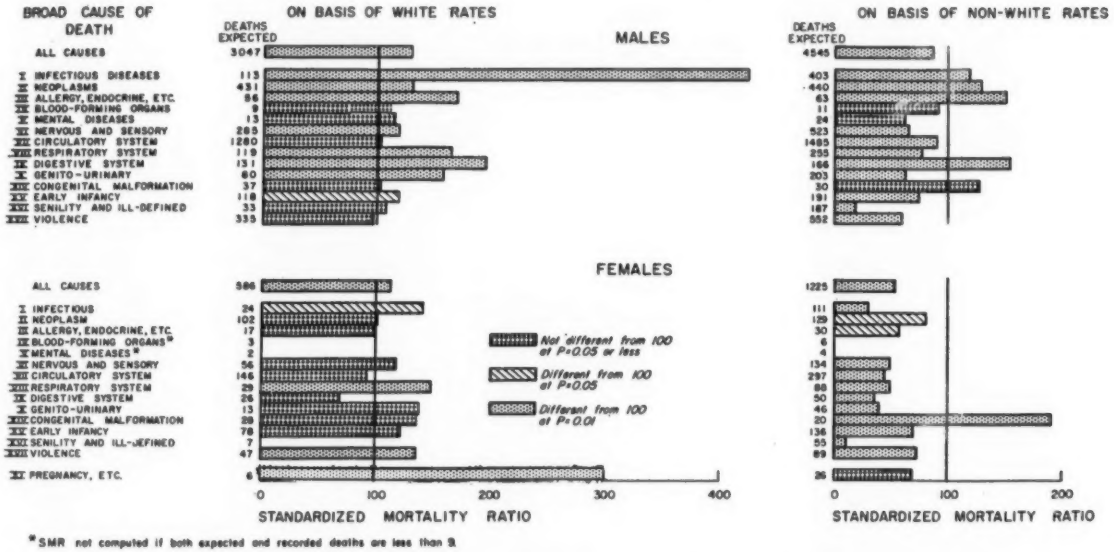
As of January 1, 1951, the median point of time for which mortality data were available, it is estimated that there were 32,647 Chinese residing in Hawaii and 120,113 residing in the Continental United States. Mortality rates, or ratios, computed for such relatively small population groups will be fairly unstable when comparisons are made by individual cancer sites, especially for the Chinese of Hawaii. It would therefore be desirable to combine the 2 groups if their mortality experience were sufficiently similar to warrant it.

Analyses directed toward this end showed that mortality for practically every cancer site was slightly lower, but not significantly lower, with the possible exception of liver, for male Chinese of Hawaii compared to those of the United States. Cancer of the liver and biliary passages, primary and not stated as primary or secondary, was only 38 percent as high among the Chinese males of Hawaii, but the difference was of borderline significance ($P = 0.04$) due to the small number of deaths involved. The

TABLE 1.—*Deaths recorded among Chinese residing in the United States during 1949–52, inclusive, by broad causes, deaths expected on the basis of age-specific rates observed in 1950 and 1951 among United States whites and nonwhites, and resultant standardized mortality ratios (SMR's)*

Broad cause of death	International list number	Males					Females				
		Number of deaths recorded	On basis of nonwhite rates		On basis of white rates		Number of deaths recorded	On basis of nonwhite rates		On basis of white rates	
			Number of deaths expected	SMR	Number of deaths expected	SMR		Number of deaths expected	SMR	Number of deaths expected	SMR
I Infective & parasitic	(001–138)	480	403	119*	113	425*	34	111	31*	24	142†
II *Neoplasm*	*(140–239)*	*566*	*440*	*129**	*431*	*131**	*103*	*129*	*80†*	*102*	*101*
Malignant	(140–205)	555	429	129*	422	132*	100	119	84	98	102
Benign	(210–239)	11	11	100	9	122	3	10	‡	4	†
III Allergy, endocrine, etc.	(240–289)	95	63	151*	56	170*	17	30	57†	17	100
IV Blood-forming organs	(290–299)	10	11	91	9	111	6	6	‡	3	‡
V Mental diseases	(300–326)	15	24	62	13	115	1	4	‡	2	‡
VI Nervous and sensory	(330–398)	339	523	65*	285	119*	66	134	49*	56	118
VII Circulatory system	(440–468)	1,318	1,485	89*	1,280	103	134	297	45*	146	92
VIII Respiratory system	(470–527)	196	255	77*	119	165*	43	88	49*	29	148*
IX Digestive system	(530–587)	255	166	154*	131	195*	18	50	36*	26	69
X Genitourinary	(590–637)	126	203	62*	80	158*	18	46	39*	13	138
XI Pregnancy and puerperium	(640–689)	—	—	—	—	—	18	26	69	6	300*
XII Skin and cellular tissue	(690–716)	3	5	‡	2	‡	1	2	‡	1	‡
XIII Bones and organs of movement	(720–749)	5	7	‡	5	‡	1	2	‡	1	‡
XIV Congenital malformation	(750–759)	38	30	127	37	103	38	20	190*	28	136
XV Early infancy	(760–776)	141	191	74*	118	119†	95	136	70*	78	122
XVI Senility and ill-defined	(780–795)	36	187	19*	33	109	6	55	11*	7	‡
XVII Violence	(800–999)	327	552	59*	335	98	64	89	72*	47	136*
All causes	(001–999)	3,950	4,545	87*	3,047	130*	663	1,225	54*	586	113*

*Significantly different from 100 at P=0.014 or less.
†Significantly different from 100 at P=0.015 to 0.054.
‡SMR not computed if both expected and recorded deaths are fewer than 9.



TEXT-FIGURE 1.—Mortality for all causes and for conventional broad causes: SMR's for Chinese of the United States and Hawaii, 1949–52, and deaths expected among them on the basis of United States white and nonwhite rates, 1950–51.

*SMR's are not shown if both expected and recorded deaths are fewer than 9.

total cancer mortality for Chinese males of Hawaii is only 80 percent of that of the Chinese males of the United States, but the difference is not significant. For Chinese females, in contrast to the males, the mortality of residents of Hawaii is higher than that of United States residents, though not significantly higher, for practically every site. The total cancer mortality for Chinese females of Hawaii is 40 percent higher than that of the Chinese females of the United States, and this difference is of borderline statistical significance ($P=0.02$). Thus, for mortality attributed to individual cancer sites for Chinese of Hawaii compared to those of the United States, no significant differences could be demonstrated—with the possible exception that mortality due to cancer of the liver may be lower among Chinese males of Hawaii. It was therefore deemed advisable to combine the data from the 2 areas in all subsequent analyses.

Table 2 presents the number of cancer deaths by site recorded among the Chinese of Hawaii and the United States and the deaths expected among them during the same period had they been subject to the white or nonwhite mortality rates for 1950 and 1951 (*11*). The resultant SMR's are also shown in this table and, graphically, in text-figure 2 for sites which show interesting differences.

It is seen that mortality attributed to all cancer is about 25 percent greater among Chinese males than among either white or nonwhite males and that it is markedly excessive for cancer of the pharynx and liver and biliary passages, primary or not stated to be primary or secondary. There is a marked deficit in mortality due to cancer of the prostate among Chinese males compared to white or nonwhite males. There are more recorded deaths from cancer of the rectum, pancreas, and lung and other parts of the respiratory system, as well as lymphosarcoma among Chinese males than expected on the basis of mortality of either white or nonwhite males. These differences are significant, however, only when compared to nonwhites. Another interesting comparison is a significantly higher mortality rate for cancer of the stomach among Chinese males than that recorded among white males, while the rate of the nonwhites is slightly, though not significantly, lower than that of the Chinese males. Similar differences are observed for cancer of the esophagus, but in this comparison the adjusted Chinese mortality is significantly lower ($P<0.05$) than that of the nonwhites and not significantly higher than the white rate. Mortality attributed to cancer of the urinary organs is somewhat lower among Chinese males than among whites, but there is little difference between male nonwhite and Chinese rates.

The total cancer-death rate for Chinese women is not significantly different from that of white or that of nonwhite females. However, there are significantly more deaths attributed to cancer of the pharynx and lung, and to leukemia, than expected on the basis of either the white or nonwhite rates. The adjusted mortality for cancer of the liver, although numerically greater among Chinese females, is not significantly higher than that of white or nonwhite females.

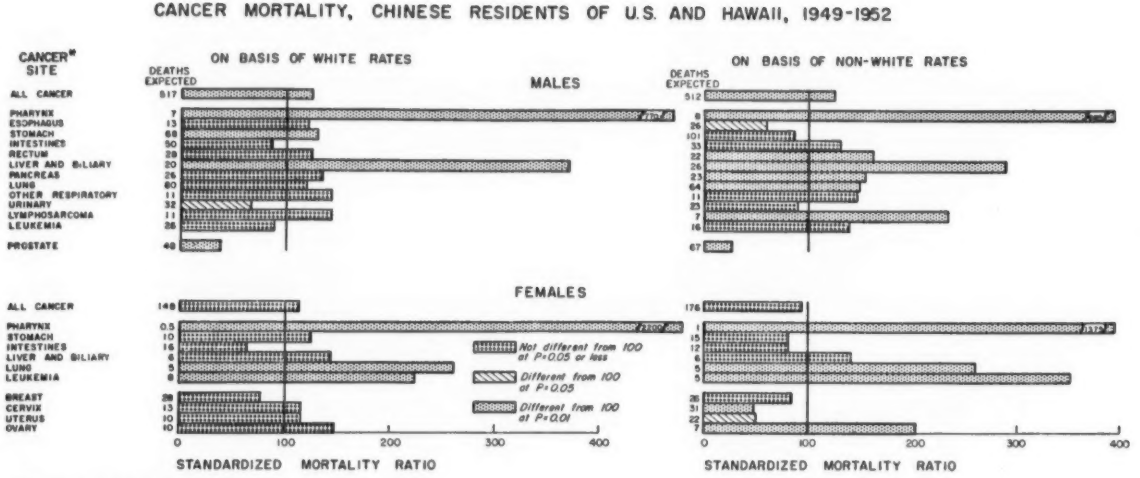
Among Chinese females, the mortality for cancer of the breast is not

TABLE 2.—*Deaths recorded among the Chinese of Hawaii and the United States during 1949–52, inclusive, expected deaths for the same period on the basis of age-specific rates for 1950 and 1951 among United States white and nonwhite males and females, and resultant standardized mortality ratios (SMR's)**

Cancer site	International list number	Male					Female				
		Number deaths recorded	Presuming U. S. nonwhite rates prevailed		Presuming U. S. white rates prevailed		Number deaths recorded	Presuming U. S. nonwhite rates prevailed		Presuming U. S. white rates prevailed	
			Number deaths expected	SMR	Number deaths expected	SMR		Number deaths expected	SMR	Number deaths expected	SMR
Cancer all sites	(*140–205*)	*643*	*511.8*	*126*†	*517.4*	*124*†	*168*	*176.2*	*95*	*147.5*	*114*
Lip, tongue, oral cavity, and pharynx	(*140–148*)	*66*	*17.5*	*377*†	*18.9*	*349*†	*14*	*2.1*	*667*†	*1.3*	*1077*†
Lip, tongue, oral cavity	(140–144)	9	9.3	97	11.5	78	3	1.3	‡	0.8	‡
Pharynx	(145–148)	57	8.2	695†	7.4	770†	11	0.8	1375†	0.5	2200†
Digestive tract and peritoneum	(*150–156A, 157–159*)	*303*	*235.9*	*128*†	*208.8*	*145*†	*42*	*47.5*	*88*	*43.2*	*97*
Esophagus	(150)	16	26.5	60§	13.2	121	—	2.0	‡	0.9	‡
Stomach	(151)	88	101.0	87	67.8	130†	12	14.8	81	9.6	125
Intestines and rectum	(*152–154*)	*78*	*54.4*	*143*†	*78.1*	*100*	*16*	*18.2*	*88*	*20.9*	*77*
Intestines	(152–153)	43	32.8	131	50.2	86	10	12.3	81	15.7	64
Rectum	(154)	35	21.6	162†	27.9	125	6	5.9	102	5.2	115
Liver and biliary passages	(*155–156A*)	*75*	*25.9*	*290*†	*20.2*	*371*†	*9*	*6.4*	*141*	*6.3*	*143*
Liver and biliary passages, primary	(155)	28	9.5	295†	8.6	326†	5	2.3	‡	3.5	‡
Liver, unstated whether primary or secondary	(156A)	47	16.4	287†	11.6	405†	4	4.1	‡	2.8	‡
Pancreas	(157)	35	22.6	155†	26.1	134	3	4.4	‡	4.4	‡
Peritoneum, other, and unspecified	(158–159)	11	5.5	200§	3.4	324†	2	1.7	‡	1.0	‡
Respiratory system	(*160–164*)	*112*	*75.3*	*149*†	*90.8*	*125*§	*13*	*5.6*	*232*†	*5.3*	*245*†
Trachea, bronchus, and lung	(162–163)	96	64.5	149†	79.6	121	12	4.6	261†	4.6	261†
Other	(160, 161, 164)	16	10.8	148	11.2	143	1	0.9	‡	0.7	‡

Other	(160, 161, 164)	16	10.8	148	11.2	143	1	0.9	‡	0.7	‡
Breast and genitourinary organs	*(170–181)*	*44*	*95.9*	*46†*	*86.1*	*51†*	*65*	*92.7*	*70†*	*65.4*	*99*
Breast	(170)	—	1.4	‡	1.1	‡	22	26.2	84	28.5	77
Genital organs	*(171–179)*	*23*	*71.3*	*32†*	*53.1*	*43†*	*40*	*61.5*	*65†*	*32.6*	*123*
Uterus	*(171–174)*	—	—	—	—	—	*26*	*53.2*	*49†*	*22.4*	*116*
Cervix	(171)	—	—	—	—	—	15	31.0	48†	12.9	116
Other and unspecified parts of uterus	(172–174)	—	—	—	—	—	11	22.2	50§	9.5	116
Ovary	(175)	—	—	—	—	—	14	6.9	203†	9.5	147
Other and unspecified female genital organs	(176)	—	—	—	—	—	—	1.4	‡	0.8	‡
Prostate	(177)	18	66.7	27†	48.0	38†	—	—	—	—	—
Testis, other, and unspecified male	(178–179)	5	4.6	‡	5.1	‡	—	—	—	—	—
Kidney, urinary bladder, and other urinary	(180–181)	21	23.2	91	31.9	66§	3	5.0	‡	4.3	‡
Other and unspecified sites	*(156B, 165, 190–199)*	*69*	*51.9*	*133§*	*60.9*	*113*	*12*	*18.2*	*66*	*17.8*	*67*
Skin	(190–191)	3	3.6	‡	9.8	31	1	1.3	‡	2.1	‡
Brain and other nervous system	(193)	8	6.2	‡	15.4	52	4	2.5	‡	4.5	‡
Bone and connective tissue	(196–197)	7	6.7	‡	7.7	‡	2	2.1	‡	2.0	‡
Other	(156B, 165, 192, 194, 195, 198–199)	51	35.4	144†	28.7	178†	5	12.3	41§	9.2	54
Leukemia and lymphoma	*(200–205)*	*46*	*33.2*	*139§*	*51.8*	*89*	*21*	*9.2*	*228†*	*14.4*	*146*
Lymphoma and mycosis fungoides	*(200–203, 205)*	*26*	*18.8*	*138*	*25.7*	*101*	*3*	*4.8*	‡	*5.9*	‡
Lymphosarcoma and reticulosarcoma	(200)	16	6.8	235†	11.2	143	2	1.6	‡	2.5	‡
Hodgkin's disease	(201)	5	5.9	‡	8.8	‡	—	1.7	‡	2.2	‡
Other lymphoma and mycosis fungoides	(202, 203, 205)	5	6.1	‡	5.8	‡	1	1.5	‡	1.2	‡
Leukemia and aleukemia	(204)	23	16.4	140	26.1	88	19	5.4	352†	8.5	224†

*Italicized figures represent totals of 2 or more listed specific cancer sites.
†Significantly different from 100 at $P = 0.014$ or less.
‡SMR not computed where both expected and recorded deaths are fewer than 9.
§Significantly different from 100 at $P = 0.015$ to 0.054.



TEXT-FIGURE 2.—Malignant neoplasms, selected sites: SMR's for Chinese of the United States and Hawaii, 1949–52, and deaths expected among them on the basis of United States white and nonwhite rates, 1950–51.

*Sites not shown are those in which both observed and expected deaths are fewer than 9.

significantly lower than that of either the nonwhites or whites. However, Chinese women have a lower mortality rate for cancer of the uterus than nonwhites, and a higher, though not significantly higher, rate than whites. The SMR's are practically the same for cervix as for fundus and unspecified parts of the uterus, in comparison with white and nonwhite female mortality. There were 14 deaths among Chinese females attributed to cancer of the ovary, fallopian tubes, and broad ligament compared to 6.9 expected on the basis of nonwhite death rates and 9.5 if white rates had prevailed.

It is interesting that although mortality for cancer of the pharynx (145–148) is markedly higher among Chinese in all comparisons, mortality attributed to cancer of the lip, tongue, salivary glands, and oral cavity (140–144) does not differ much from that of the whites or nonwhites.

Discussion

The results of the analyses of data here presented, comparing cancer mortality among Chinese of Hawaii and the United States with the mortality of whites and nonwhites of the United States, are consistent in many respects with relative-frequency data on clinical and pathologic material reported previously and reviewed by Steiner (*12*). Thus, among Chinese high mortality attributable to cancer of the pharynx and liver is borne out, as well as low mortality for cancer of the prostate. However, malignancies of the uterus are not excessive as compared to white rates and are lower among Chinese women than nonwhites. This finding is opposed to the high frequency for cancer of this site reported by others from clinical and autopsy series (*1,8,13*).

Mortality for cancer of the liver appears to be somewhat higher among male Chinese of the United States than among those of Hawaii, suggesting a possible environmental factor, but the number of deaths is too small to place much significance on the difference. This is in line, however, with possible geographic variations within China noted by Steiner (*12*).

Unfortunately no reliable mortality and population data are available from which death rates for large groups of Chinese can be computed so that they can be compared with the mortality rates of Chinese in America. Furthermore, the Chinese population of this country differs markedly from the whites or nonwhites in the proportions comprising different age groups as well as in the proportion of males to females. These factors, coupled with the factor of a relatively small Chinese population, combine to limit the reliability of comparisons of mortality among the Chinese with that of other races, especially mortality assigned to cancer sites where few deaths are recorded. Comparisons of mortality between segments of the Chinese population, *e.g.*, those of Hawaii and the Continental United States, are similarly limited.

Summary

In this study the mortality recorded among the Chinese of America is compared to that of the white and nonwhite populations of the United

States. It was observed that the adjusted death rate for all causes was higher among Chinese of the United States than among whites and lower than among nonwhites, and the same differences existed for many of the broad causes of death.

After observing that there was little difference between mortality rates for specific cancer sites among the Chinese of Hawaii and those of the United States, except for cancer of the liver among the males, the mortality experience of the 2 population segments was combined. Analysis revealed that Chinese males had a higher cancer mortality than white or nonwhite males, while no significant difference was observed for females. Among the Chinese, both sexes experienced markedly excessive mortality attributed to cancer of the pharynx. Mortality for cancer of the liver was higher among Chinese than that of either whites or nonwhites but the difference was not significant for females. There were excesses in all comparisons for mortality attributed to cancer of the lung except the comparison with white male rates. Marked deficits were observed for mortality from cancer of the prostate as compared to white or nonwhite rates. No significant difference was noted between Chinese and white or nonwhite females with respect to mortality from cancer of the breast; for mortality from uterine cancer there was no significent difference between Chinese and white rate. For the latter site, however, the mortality rate for Chinese females was only 49 percent of the nonwhite rate.

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Studies on the Protein Binding of *N*-2-Fluorenylacetamide[1]

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The demonstration by the Millers and their co-workers (*1,2*) that aminoazo dyes are bound by protein of the livers of rats fed carcinogenic azo dyes, and their suggestion that such protein binding may be an essential step in the ultimate carcinogenic process, led to the present investigation of protein binding of *N*-2-fluorenylacetamide.[4] The colorimetric diazotization methods of analysis for 2-fluorenamine and *N*-2-fluorenylacetamide (*3,4*) and for the metabolite *N-2*-(7-hydroxyfluorenyl) acetamide (*5*) would be expected to detect free rather than bound fluorenamine compounds. It was decided, therefore, to administer the carcinogen tagged with isotopic nitrogen and to look for N^{15} enrichment of proteins isolated from the tissues. It has been observed that N^{15} of the amino group of N^{15}-2-fluorenylacetamide and C^{14} of *N-2*-fluoren-9-C^{14}-ylacetamide have essentially the same distribution in the tissues and excreta of the rat following gastric administration, suggesting that the molecule remains intact, during its metabolism in the animal body, with respect to the 2 and 9 positions (*6,7*). Moreover, no evidence has been found of the loss of more than a trace of nitrogen from 2-fluorenylacetamide during its passage through the body (*7*).[5] The enrichment, therefore, of tissue proteins with isotopic nitrogen following administration of the tagged carcinogen would offer suggestive, although not absolute, proof of the binding of fluorenylacetamide or a structurally related metabolite. Concurrent with this investigation, strong evidence of such a binding has been furnished in the finding of bound radioactivity in tissue proteins following studies with *N*-2-fluoren-9-C^{14}-ylacetamide (*9,10*).

Since 2-fluorenylacetamide has a wide distribution in the body of the rat (*6,7,11*) and induces a large variety of tumor types, protein fractions from a number of different organs and tissues, including primary induced

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[4] Other papers from this laboratory have referred to this compound as 2-acetylaminofluorene (2-AAF). Hereafter the name recommended by *Chemical Abstracts*, *N*-2-fluorenylacetamide, will be used.

[5] Excess isotopic nitrogen was not present in urea isolated from the N^{15}-rich urine of rats fed N^{15}-2-fluorenylacetamide. Ammonia aerated from the 0-25- and 24-48-hour samples of urine of 3 rats following N^{15}-2-fluorenylacetamide administration contained a trace to 0.05 percent of the total N^{15} of the urine, and no isotopic nitrogen was detected in the ammonia from the 48-72-hour samples (*8*).

and transplanted liver tumors, were analyzed for isotopic nitrogen. Most of the analyses, however, were made on liver proteins from young adult male Buffalo rats following repeated administration of N^{15}-2-fluorenylacetamide. For comparison, analyses were made on liver proteins of rats given equimolar doses of N^{15}-2-fluorenyldiacetamide, 2-nitro-N^{15}-fluorene, and of the relatively noncarcinogenic N^{15}-4-fluorenylacetamide (*12,13*). Binding of isotopic nitrogen by the liver proteins of female Buffalo strain rats, of A × C/9935 (Irish) strain rats, males and females, and of 2 guinea pigs was investigated also under the same experimental conditions, following administration of N^{15}-2-fluorenylacetamide. In addition, a preliminary investigation was made of the possibility of protein binding of N^{15} of some of these compounds *in vitro*.

Materials and Procedures

The aminofluorene derivatives enriched with 60 percent N^{15} [6] were administered repeatedly in propylene glycol by stomach tube and/or were incorporated in a diet of natural foodstuffs, #222 (*12*), and fed *ad libitum*. In all but one group of rats that received N-2-fluorenylacetamide incorporated in the food for periods longer than 2 weeks nonisotopic material was used up to the last 2 weeks, when the N^{15}-tagged carcinogen was substituted.

In the first experiment rats were killed at time intervals of 6 and 24 hours and 1 and 5 weeks following the last of thirteen 10 mg. doses of N^{15}-2-fluorenylacetamide per 100 gm. body weight administered by stomach tube at intervals of 2 to 3 days. Thereafter, several schedules of administration were followed in an effort to increase the concentration of isotopic nitrogen bound by the proteins. The rats in the later experiments were killed 18 to 24 hours after receiving the carcinogen, with 2 exceptions of 48-hour intervals. One group of animals was fed N^{15}-2-fluorenylacetamide through the time of the appearance of tumors of the liver and/or the mammae. Histologic sections made from the tumors were examined and the tumors were classified by Dr. Clarence A. Velat,[7] formerly of the Laboratory of Pathology of the National Cancer Institute.

Preparation of crude protein fractions.—The method used for the separation and washing of crude protein fractions followed, with some modifications, the acetate buffer extraction-heat coagulation procedure of Miller and Miller. The animals were anesthetized with ether, bled to death by cardiac puncture or decapitation, and the livers were perfused with 0.85 percent NaCl. In the first group of 8 rats whole livers were used. Thereafter, 4 gm. samples of liver and, where tissues other than the liver were used, 4 gm. of abdominal muscle, and ≦ 4 gm. samples of tumors were extracted separately. The individual livers were often large enough to provide 2 samples. The briefly water-rinsed small intestine, which weighed approximately 4 gm., and the kidneys from each rat were treated separately, whereas the heart, spleen, sample of abdominal skin,

[6] The N^{15}-enriched fluorene derivatives were prepared for us by Dr. F. E. Ray and his associates of the Cancer Research Laboratory, University of Florida, Gainesville, Fla. (*14-16*).

[7] *Present address:* Providence Hospital, Detroit, Mich.

lungs, and brain were pooled with the corresponding material of another rat that had received the same treatment. The weighed samples were minced and homogenized in distilled water, using glass homogenizers. The homogenates were diluted with acetate buffer, the final mixture per gm. of tissue containing 4 volumes of water and 5 volumes of 1 *M* sodium acetate buffer of *p*H 5. Extraction, with occasional stirring, was continued in an ice bath for 1 to 1.5 hours before centrifugation. The supernatant solution, combined with a first 0.5 *M* acetate buffer wash of the insoluble residue, was boiled on a hot plate for 3 minutes with constant stirring to coagulate the acetate-soluble proteins. The second acetate wash of the insoluble residue had been found to contain little, if any, heat-coagulable material. The insoluble residue was washed 2 more times with acetate buffer, using 10 volumes per gm. of original tissue for each wash. The coagulated acetate-soluble protein fraction was centrifuged and washed 2 times with acetate buffer. After washing with buffer the coagulated acetate-soluble protein fractions and the insoluble residues were washed 2 times with 95 percent ethanol at room temperature, after which they were placed in filter-paper thimbles and extracted with 95 percent ethanol continuously for 48 hours in a Soxhlet apparatus, then dried in a desiccator over H_2SO_4.

Determination of total and isotopic nitrogen.—The protein fractions and representative samples of the acetate and alcohol washes were analyzed for total and isotopic nitrogen. The nitrogen content was determined by a modified micro-Kjeldahl method and the titrated sample of ammonia was concentrated, oxidized to nitrogen gas, and prepared for analysis as previously described (7). The $N^{15} : N^{14}$ ratio of the nitrogen gas was determined in a Consolidated-Nier mass spectrometer.[8] Duplicate and sometimes triplicate samples of the fractions (with exceptions due to lack of material) were analyzed for total ammonium nitrogen, and each sample of total ammonium nitrogen usually provided 2 samples of nitrogen gas for duplicate determinations of the $N^{15} : N^{14}$ ratios.

Control values were obtained for corresponding materials from untreated rats. The atom percent excess N^{15} was calculated by subtraction of the atom percent air-free N^{15} of reference-standard tank nitrogen from the atom percent air-free N^{15} of the experimental material. To determine the amount of excess N^{15} present in the sample as a result of administration of the carcinogen, the atom percent air-free excess N^{15} (over that of tank nitrogen) of the corresponding fraction from control rats was subtracted from the atom percent air-free excess N^{15} (over that of tank nitrogen) of the material from the treated animal. The difference, representing enrichment relative to the normal concentration of isotopic nitrogen in the tissue, was converted, for comparison, in one experiment, to the equivalent weight of the administered carcinogen which was responsible for the enrichment.

Stability of the union of N^{15} *with the protein material.*—To test the

[8] The titrated, concentrated samples containing indicator that have been used for the determination of total nitrogen give the same ratios of isotopic to total nitrogen as duplicate samples that do not contain the indicator of mixed methyl red and bromcresol green.

tightness of the binding of isotopic nitrogen by the proteins, liver fractions from 21 of the rats, after removal of portions for analysis, were re-extracted twice each, successively, with hot 50 percent ethanol, hot 92 percent acetone, and finally with ethyl ether. They were then reanalyzed for total and isotopic nitrogen. Samples of pooled crude proteins from livers were extracted 3 times with rubbing and standing in ethyl ether, hot acetone, hot pyridine, hot glacial acetic acid, hot 0.1 *N* HCl, or warm 0.1 *N* NaOH. Much of the material dissolved in the NaOH. The supernatant solution from the centrifuged alkaline extract was treated with an equal volume of 6 percent trichloroacetic acid and the resulting precipitate was centrifuged, washed with alcohol, dried, and analyzed. Samples of pooled N^{15}-enriched proteins from the livers of 4 rats fed N^{15}-2-fluorenylacetamide were extracted with 20 percent aqueous, followed by 20 percent alcoholic, mercaptoethanol, for 4 hours at room temperature, with stirring and rubbing, and finally dried with ethanol and ether. Aqueous suspensions of isolated proteins and acetate buffer homogenates of liver from rats given N^{15}-2-fluorenylacetamide were dialyzed for 72 hours in running water at a controlled temperature of 9° C. After dialysis the suspended proteins were centrifuged and dried for analysis, and insoluble and coagulated soluble fractions were prepared from the dialyzed homogenates. Other samples of the pooled material were extracted with 20 percent Tween 80 or with aqueous 6.6 *M* urea, after which they were dialyzed in cold running water for 70 hours, centrifuged, and dried after washing with ethanol and ether. Some of the pooled liver proteins and some protein fractions of primary tumors and of a primary hepatoma (all of which appeared during the feeding of N^{15}-2-fluorenylacetamide) were refrigerated overnight in 0.05 *N* NaOH. After centrifugation, the supernatant solutions were adjusted with HCl to a slightly acid *p*H (approximately 5.5) to the appearance of turbidity. The alkali-soluble reprecipitated fractions and the alkali-insoluble residues were washed with alcohol and with ether, dried in a vacuum desiccator, and analyzed for total and isotopic nitrogen.

To investigate the solubility behavior of the N^{15}-containing material present in the proteins 2 samples each of the coagulated and the insoluble fractions from livers were digested for 18 hours by refluxing in 50 percent alcoholic KOH. The alcohol was distilled off at reduced pressure and the digested residues were taken up in small amounts of water and extracted with ether, repeatedly, in separatory funnels. Analyses were made of the evaporated ether residues and the aqueous extracts. Further fractionation of the alcoholic-KOH digests of proteins from the liver and small intestine of rats given N^{15}-2-fluorenylacetamide was attempted by neutralization of the digests with HCl, removal of the water-alcohol layer by distillation at reduced pressure, and extraction of the salt residue with ethanol. The ethanol residue, after evaporation of the extract in an atmosphere of nitrogen, and the alcohol-extracted salt residue were suspended in small amounts of water and extracted with ether. The final residues and water and ether extracts were analyzed separately.

In vitro studies.—Homogenates and slices of rat liver were incubated with N^{15}-2-fluorenylacetamide, N^{15}-2-fluorenamine, or 2-nitro-N^{15}-fluorene. The substrates were prepared by stirring the carcinogens in boiling 0.5 *M* acetate buffer at *p*H 5.2 or 5.8 and filtering while hot. Nine ml. of the warm filtered solutions were transferred to tubes into which were introduced the homogenates of 1 gm. samples of tissue in 1 ml. of H_2O. Control tubes contained the buffer only as substrate. After incubation for 3.5 hours at 37° C. with occasional stirring or with constant rocking, the coagulated acetate-soluble protein fractions and the insoluble residues were isolated, washed, analyzed, and re-extracted in the same manner as those fractions from the livers of carcinogen-fed rats. For comparison, homogenates of rat kidney and homogenates and slices of transplanted liver tumors from rats, guinea-pig liver, and solutions of crystalline bovine-serum albumin were also used.

Results

The crude protein fractions.—Except for samples from the spleens and occasional fractions isolated from blood, which were tan, the crude, dry, coagulated acetate-soluble proteins were white and powdery and the insoluble residues consisted of both fibrous and powdered material. The nitrogen contents of the fractions from all the tissues showed little variation, having a mean of 14.3 percent (± 0.025 S.E.). Both fractions lost 2 to 3 percent of their total nitrogen during re-extraction with hot organic solvents but there was little, if any, change in the concentration of isotopic nitrogen.

The combined weights of the 2 protein fractions from rat liver averaged 16 percent of the wet weight of the liver compared with an average of 11.8 percent for the single dry powder reported by the Millers (*1*). As would be expected there was considerable variation among different tissues in the amounts of crude protein isolated—from 8 to 22 percent of the wet weight of the tissue. The dry soluble protein varied from 3 percent of the total protein for skin to 46 percent for the primary hepatoma.

No ash (sulfate method) was detectable in either the coagulated-soluble or the acetate-insoluble proteins from liver of rats. The total phosphorus (*17*) contents of the isolated crude liver proteins were 0.209 percent for the soluble and 0.563 to 0.584 percent for the insoluble fractions.[9]

N^{15} *Enrichment of protein fractions from the liver.*—Table 1 shows the amount of variation found in the concentrations of isotopic nitrogen in the isolated protein fractions from the livers of control rats and the slightly but consistently greater enrichment of the corresponding materials following the final dose of repeated administrations of N^{15}-2-fluorenylacetamide 6 and 24 hours and 1 week before the livers were removed for analysis. Text-figure 1 illustrates the decrease in concentration of excess isotopic nitrogen in the crude proteins with an increasing length of time between the last administration of the carcinogen and the removal of

[9] Appreciation is expressed to Mrs. R. J. Koegel and to Mr. J. F. Williams for the analyses of ash and phosphorus.

the liver for analysis. No enrichment was detectable after an interval of 2 weeks.

TABLE 1.—*Effect of time on concentration of isotopic nitrogen in crude protein fractions from livers of rats following gastric administration of* N^{15}*-2-fluorenylacetamide*

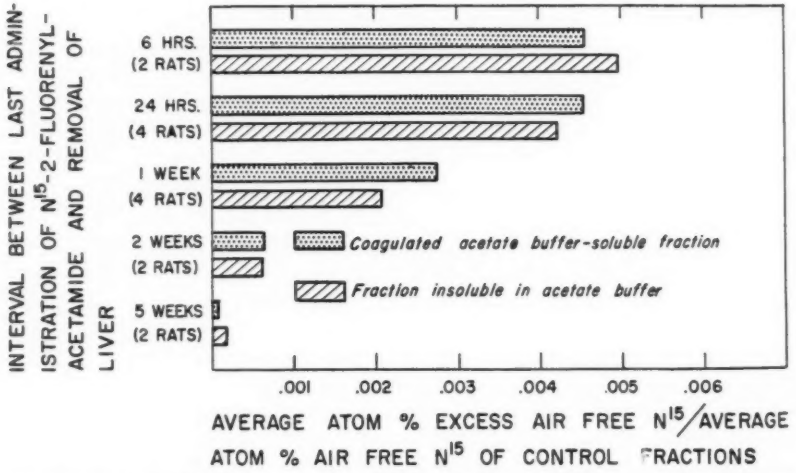
No. of rat	Interval between final dose and removal of liver	Atom percent excess N^{15} *	
		Coagulated acetate-soluble fraction	Acetate-insoluble residue
14, 29–32, 97†	Controls	.00695±.00014	.00675±.00028
114, 115†	"	.00612±.00042	.00570±.00060
7‡	5 weeks	.00680	.00628
8‡	"	.00634	.00649
109§	2 weeks	.00750	.00695
113§	"	.00685	.00675
108§	1 week	.00906	.00804
112§	"	.00900	.00816
1‡	"	.00966	.00872
2‡	"	.00936	.00831
110§	24 hours	.01172	.01078
111§	"	.01123	.01092
5‡	"	.01053	.01040
6‡	"	.01093	.00964
3‡	6 hours	.01174	.01140
4‡	"	.01050	.01096

*Atom percent excess N^{15} represents the atom percent excess air-free N^{15} of the sample over that of the tank nitrogen which was used throughout as a reference standard. Each figure is the average of values for 2-3 different analyses. The duplicate values differed from each other from 0 to 10 percent. Values for control animals are averages ± S.E. of extreme values.

†Control rats received no *N*-2-fluorenylacetamide, and tissues were analyzed concurrently with those from treated animals.

‡Rats 1 to 8 received 13 doses each (2-3 weekly) of 10 mg. per 100 gm. body weight of N^{15}-2-fluorenylacetamide during a period of 5 weeks.

§Rats 108 to 113 received 6 doses each during 2 weeks and were run with controls 114 and 115 2 years after rats 1 to 8, 14, and 29 to 32.


INTERVAL BETWEEN LAST ADMINISTRATION OF N15-2-FLUORENYLACETAMIDE AND REMOVAL OF LIVER
6 HRS. (2 RATS)
24 HRS. (4 RATS)
1 WEEK (4 RATS)
2 WEEKS (2 RATS)
5 WEEKS (2 RATS)
Coagulated acetate buffer-soluble fraction
Fraction insoluble in acetate buffer
.001
.002
.003
.004
.005
.006
AVERAGE ATOM % EXCESS AIR FREE N15/AVERAGE ATOM % AIR FREE N15 OF CONTROL FRACTIONS

TEXT-FIGURE 1.—Decrease in excess isotopic-nitrogen content of crude proteins from livers of rats following final dose of N^{15}-2-fluorenylacetamide.

Table 2 contains additional data from the first group of rats, in which whole livers were used for the isolation of the crude protein fractions. The amounts of total nitrogen of the soluble and the insoluble fractions were reasonably constant for the livers of all the animals. The total amounts of excess isotopic nitrogen, expressed for convenience as μg. of fluorenylacetamide present in both protein fractions 1 week after the last dose of N^{15}-2-fluorenylacetamide, are less than in the corresponding fractions from the rats that were killed 6 and 24 hours after the final dose of the carcinogen. There was a steady decrease in the amounts of isotopic nitrogen, presumably unbound by protein, in the acetate and alcohol washes of the protein fractions from the livers of the rats that were killed 6 hours, 24 hours, and 1 week, respectively, after receiving the final dose of N^{15}-2-fluorenylacetamide.

TABLE 2.—*Recoveries of excess isotopic nitrogen from rat-liver proteins and washes following administration of* N^{15}*-2-fluorenylacetamide**

No. of rat	Hours between final dose and removal of liver	Total fractions from whole livers							
		Coagulated acetate-soluble protein fractions		Acetate-insoluble residues		Supernatant acetate and acetate washes		Alcohol washes	
		Total nitrogen (mM)	N^{15} as FAA† (μg.)	Total nitrogen (mM)	N^{15} as FAA† (μg.)	Total nitrogen (mM)	N^{15} as FAA† (μg.)	Total nitrogen (mM)	N^{15} as FAA† (μg.)
3	6	2.9	49	8.0	128	3.2	72	0.4	75
4	6	3.0	37	8.1	116	3.5	52	0.4	62
5	24	3.5	44	8.0	98	3.2	24	0.4	18
6	24	3.2	45	8.1	77	3.1	23	0.4	20
1	168	3.1	29	9.3	56	2.9	5	0.9	9
2	168	3.1	25	9.0	41	2.6	9	1.1	11

*Ten mg. of N^{15}-2-fluorenylacetamide per 100 gm. body weight was administered to each rat by stomach tube 2-3 times weekly (13 doses) for a period of 5 weeks.

† The atom percent excess of air-free N^{15} over that of corresponding materials from control rats was used to calculate the amounts of N^{15} to be converted to equivalent weights of *N*-2-fluorenylacetamide as follows:

$$mM\ N \times \frac{\text{atom \% excess } N^{15}}{100} \times \frac{100}{60\ (a)} \times 223.262\ (b) \times 1{,}000 = \text{calculated micrograms of } N\text{-2-fluorenylacetamide (FAA).}$$

(a) 60 = atom percent excess N^{15} of N^{15}-2-fluorenylacetamide.

(b) 223.262 = m. w. of fluorenylacetamide.

The results of efforts to increase the isotopic-nitrogen contents of the crude protein fractions of the liver by varying the schedule of administration of N^{15}-2-fluorenylacetamide are summarized in table 3. The highest concentration of isotopic nitrogen was obtained when rats received by stomach tube 13 or 6 feedings, 10 mg. per 100 gm. body weight, and were killed at 6 or 24 hours after the final dose. The toxicity of 2-fluorenylacetamide made undesirable the use of repeated, larger, or more frequent doses than those given in the first experiment.

Although the ratio of the recovered N^{15} to the total nitrogen content of the fraction was usually a little greater in the coagulated than in the insoluble protein fraction from the same sample of liver, the total amount

TABLE 3.—*Concentration of isotopic nitrogen in protein fractions from livers of rats following different schedules of administration of N^{15}-2-fluorenylacetamide**

Number of rats	Schedule of administration of N^{15}-2-FAA			Variations, averages, and standard errors in N^{15} concentrations†	
	Route‡	Dose		Coagulated acetate-soluble fractions (*atom percent excess* N^{15})	Acetate-insoluble residues (*atom percent excess* N^{15})
		Schedule§	Amount‖ (*mg.*)		
8 Controls	—	—	—	.00570—.00740 (.00673±.00021)	.00510—.00750 (.00649±.0003)
4	S.T.	6 or 13	10 mg./100 gm.	.01053—.01172 (.01110±.00030)	.00964—.01092 (.01043±.00032)
1	S.T.	1	20 mg./100 gm.	.00879	.00853
10¶	Food	5.5–6 months	2.37	.00682—.01008 (.00850±.000074)	.00561—.00960 (.00787±.000085)
3	Food	40 days	2.20	.00832—.01100 (.00964±.00044)	.00745—.01129 (.00878±.00064)
2	Food	8 days	1.76	.00899—.00967 (.00939±.00017)	.00838—.01002 (.00935±.00041)
3**	Food followed by S.T.	3.5–4.0 months 3	2.70 10 mg./100 gm.	.00833—.01090 (.00906±.00026)	.00655—.00881 (.00799±.000205)
4**	Food	3.5–4.2 months 2–3 weeks	2.60 4.60	.00652—.00875 (.00787±.00013)	.00691—.01042 (.00832±.00027)

*These rats were killed 24 to 30 hours after the last administration of N^{15}-2-fluorenylacetamide (FAA) by stomach tube and 16 to 20 hours after removal of N^{15}-2-fluorenylacetamide-containing food.

†Values represent all of the data from duplicate or triplicate analyses of 1 or 2 samples from the liver of each rat. In parentheses: mean ± S.E.

‡S.T.—by stomach tube; food—incorporated in diet #222.

§ When *N*-2-fluorenylacetamide was incorporated in the food, administration was *ad libitum*. When feeding was carried over extended periods *N*-2-fluorenylacetamide was given up to the final 2 to 3 weeks when N^{15}-2-fluorenylacetamide was substituted.

‖ Dosage as mg. *N*-2-fluorenylacetamide per 100 gm. body weight or as average daily intake in mg. when incorporated in food.

¶ Five of these rats were males, 3 of which had tumor nodules scattered throughout the liver. One had a few scattered liver nodules and 1 had a cystic liver. The 5 females had mammary tumors and 1 had a cystic liver.

**These livers were granular in appearance and contained scattered white nodules.

of isotopic nitrogen was greater in the insoluble residue, which weighed from 65 to 75 percent of the sum of the weights of the 2 fractions.

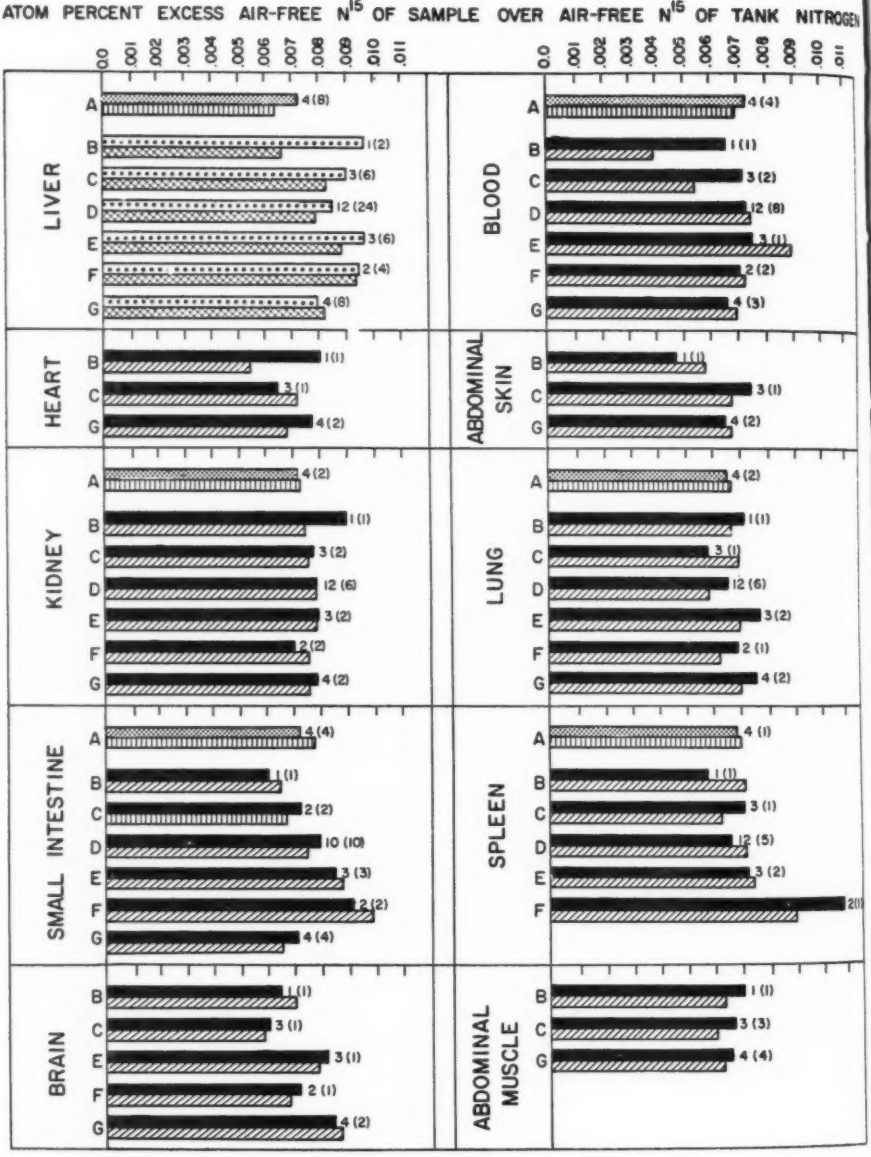
N^{15} *Enrichment of protein fractions of miscellaneous tissues.*—The number of rats and samples, the tissues that were analyzed, and the results of the analyses are summarized in text-figures 2 and 3. The N^{15} contents of fractions from the livers of the same rats are given in the text-figures for comparison with those from the other tissues. It is unfortunate that some of these rats were selected for analysis of miscellaneous tissues since subsequent analyses showed that the liver material from 5 of these groups that were given N^{15}-2-fluorenylacetamide incorporated in the diet had less enrichment than the livers of rats given larger doses of the carcinogen by stomach tube. Included in this series also was the only rat in our experience from which a fraction from the liver, the buffer-insoluble fraction from 1 rat, B, showed no evidence of enrichment following stomach-tube administration of the tagged carcinogen. The fractions isolated from liver usually had greater ratios of isotopic to total nitrogen than those fractions from any of the other tissues isolated from the same rats. The exceptions found were from brain tissue of group G, small intestine of group F, the soluble fractions of the spleens from group F and 1 sample of fibrin. There is evidence of some slight binding of isotopic nitrogen in the 1 discrete primary hepatoma and in the soluble fractions from subcutaneously and intramuscularly transplanted hepatoma 3683, transplant generation 38, and cholangioma 3924A, generation 35. Re-extraction of the materials from the transplanted liver tumors with hot alcohol, acetone, and ether did not influence the small amounts of isotopic nitrogen present.

N^{15} *Enrichment of washes of protein fractions.*—The acetate washes of fractions from the livers, small intestines, and transplanted hepatoma 3683, and occasionally from the kidneys of rats that had received N^{15}-2-fluorenylacetamide by stomach tube or feeding within 24 hours of sacrifice contained traces to small amounts of excess isotopic nitrogen, while the alcohol washes of these materials, and in several instances the alcohol washes of the fractions from blood and from lungs, contained significantly larger amounts of excess isotopic nitrogen than the washes of corresponding materials from control rats. The $N^{15} : N^{14}$ ratios of the alcohol washes from liver, intestine, and kidneys were sometimes 3 to 10 times as great as the maximum ratios found in isolated liver fractions. The washes from fractions isolated from muscle, skin, and spleen, and usually from the blood, were not enriched. The excess isotopic nitrogen that was present in acetate and alcohol washes was presumably in material that was not bound by protein.

N^{15}-*2-Fluorenyldiacetamide and 2-nitro-*N^{15}-*fluorene.*—After administration of these fluorene derivatives to 2 rats each, in 4 doses equivalent in moles to 10 mg. *N*-2-fluorenylacetamide per 100 gm. body weight, the same maximum amount of isotopic-nitrogen enrichment of the crude fractions from the livers was observed as with N^{15}-2-fluorenylacetamide.

N^{15}-*4-Fluorenylacetamide.*—Unlike the protein materials from the rats given N^{15}-2-fluorenylacetamide, the corresponding fractions from the

the final 2 to 3 weeks when N^{14}-2-fluorenylacetamid^{15}e was substituted.
‖ Dosage as mg. *N*-2-fluorenylacetamide per 100 gm. body weight or as average daily intake in mg. when incorporated in food.
¶ Five of these rats were males, 3 of which had tumor nodules scattered throughout the liver. One had a few scattered liver nodules and 1 had a cystic liver. The 5 females had mammary tumors and 1 had a cystic liver.
** These livers were granular in appearance and contained scattered white nodules.


ATOM PERCENT EXCESS AIR-FREE N15 OF SAMPLE OVER AIR-FREE N15 OF TANK NITROGEN
0.0 .001 .002 .003 .004 .005 .006 .007 .008 .009 .010 .011
LIVER
A 4 (8)
B 1 (2)
C 3 (6)
D 12 (24)
E 3 (6)
F 2 (4)
G 4 (8)
BLOOD
A 4 (4)
B 1 (1)
C 3 (2)
D 12 (8)
E 3 (1)
F 2 (2)
G 4 (3)
HEART
B 1 (1)
C 3 (1)
G 4 (2)
ABDOMINAL SKIN
B 1 (1)
C 3 (1)
G 4 (2)
KIDNEY
A 4 (2)
B 1 (1)
C 3 (2)
D 12 (6)
E 3 (2)
F 2 (2)
G 4 (2)
LUNG
A 4 (2)
B 1 (1)
C 3 (1)
D 12 (6)
E 3 (2)
F 2 (1)
G 4 (2)
SMALL INTESTINE
A 4 (4)
B 1 (1)
C 2 (2)
D 10 (10)
E 3 (3)
F 2 (2)
G 4 (4)
SPLEEN
A 4 (1)
B 1 (1)
C 3 (1)
D 12 (5)
E 3 (2)
F 20
BRAIN
B 1 (1)
C 3 (1)
E 3 (1)
F 2 (1)
G 4 (2)
ABDOMINAL MUSCLE
B 1 (1)
C 3 (3)
G 4 (4)
Coagulated acetate buffer-soluble proteins, control rats
Acetate buffer-insoluble proteins, control rats
Coagulated acetate buffer-soluble proteins, liver, N15-2-fluorenylacetamide rats
Acetate buffer-insoluble proteins, liver, N15-2-fluorenylacetamide rats
Coagulated acetate buffer-soluble proteins, miscellaneous tissues, N15-2-fluorenylacetamide rats
Acetate buffer-insoluble proteins, miscellaneous tissues, N15-2-fluorenylacetamide rats

livers of 4 rats that received 6 doses of N^{15}-4-fluorenylacetamide by stomach tube during a period of 2 weeks showed little, if any, evidence of binding of isotopic nitrogen. The alcohol washes from these fractions were enriched.

Protein fractions from guinea pig.—Although the guinea pig appears to resemble the rat in the rapid excretion of diazotizable and hydroxylated metabolites (*8*) enrichment with isotopic nitrogen of the protein fractions of the livers of 2 pigs was even less than that in the rat after administration by stomach tube of 1 dose to the first pig and 6 doses of 10 mg. N^{15}-2-fluorenylacetamide per 100 gm. of body weight to the second. The fractions from the livers of 2 untreated pigs varied from 0.00425 to 0.00493 atom percent excess N^{15} over that of tank nitrogen, values that are lower than those of rat liver. In the fractions from pigs given carcinogen the extreme values were 0.00501 to 0.00722 with no apparent difference as a result of 1 or 6 doses. The average enrichment of 3 samples of liver from the first pig and 4 samples from the second was 0.0018 for the soluble and 0.0019 for the insoluble fractions, relative to the liver fractions of the control pigs. Bile, bladder, urine, and a sample of stomach contents of the second pig at the time of removal of the liver had atom percent excesses of isotopic nitrogen over tank nitrogen of 2.98, 0.88, and 0.11, respectively, while the alcoholic washes of liver proteins showed the same small degree of enrichment as corresponding washes from rats. The guinea pigs would not eat the food used for rats so the 2 species were compared on different diets, the guinea pigs having been fed rabbit pellets and cabbage.

Firmness of the binding of the excess isotopic nitrogen to protein material.—Successive extractions of the materials from the livers of rats with the organic solvents, as described earlier, failed to result in any decrease in

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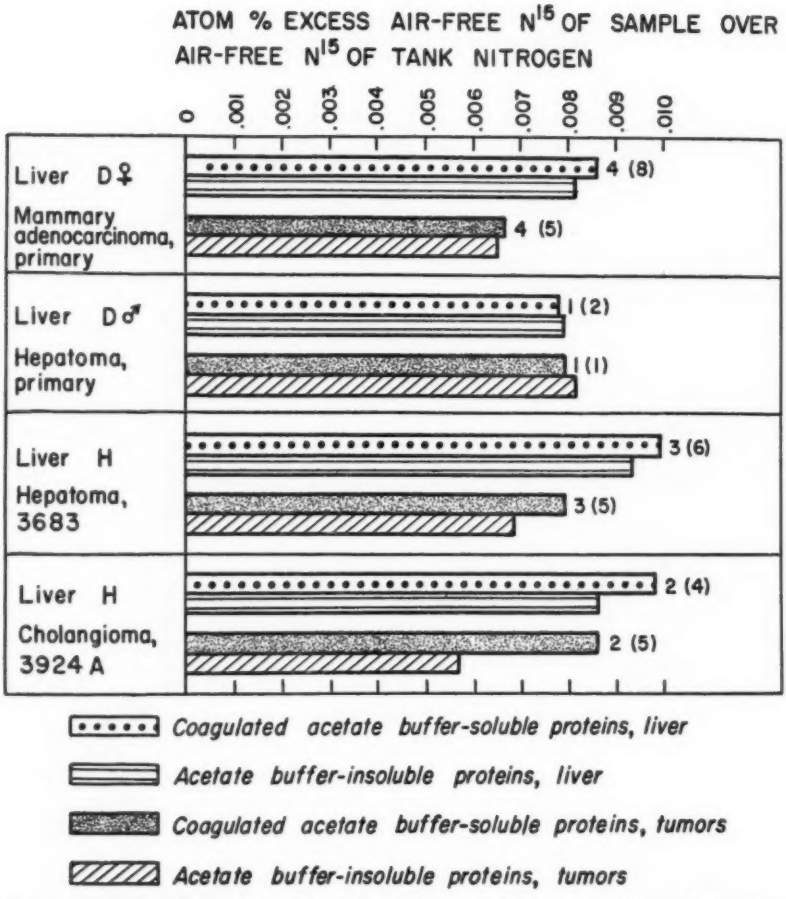
TEXT-FIGURE 2.—Comparison of isotopic-nitrogen contents of crude protein fractions of miscellaneous tissues with those of liver fractions from the same control rats or the same rats given N^{15}-2-fluorenylacetamide.

Numbers indicate number of rats used. Numbers in parentheses: number of samples of tissues fractionated into soluble and insoluble proteins.

Letters designate groups of rats or the rat of Buffalo strain from which tissues were obtained and for which dosages follow:

Group A: No isotopic nitrogen was administered to control rats.
B: 10 mg. per 100 gm. body weight by stomach tube 10 times in 1 month.
C: 0.025 percent incorporated in diet for 110 days, followed by 10 mg. per 100 gm. body weight by stomach tube 3 times in 10 days.
D: 0.025 percent incorporated in diet for 5 to 6 months.
E: " " " " " " 40 days.
F: " " " " " " 8 days.
G: " " " " " " 110 days, followed by 0.05 percent for 7 to 21 days.



TEXT-FIGURE 3.—Comparison of isotopic-nitrogen contents of crude protein fractions from tumors and from the livers of the tumor-bearing rats following administration of N^{15}-2-fluorenylacetamide.

Numbers indicate number of rats used. Numbers in parentheses: number of samples of tissue fractionated into soluble and insoluble proteins.

Letters designate groups of rats or the rat from which the tissues were obtained, for which the strains and dosages follow:

Group D of Buffalo strain: 0.025 percent incorporated in diet for 5 to 6 months.

H of A × C/9935 (Irish) strain: 10 mg. per 100 gm. body weight by stomach tube 4 times in 10 days. Subcutaneous and intramuscular transplants.

Histologic examination of tumors by Dr. Clarence A. Velat.

the N^{15} content. The concentrations of N^{15} in the recovered trichloroacetic acid precipitates from warm NaOH solutions were the same as in the original material. Dialysis of water suspensions of the isolated liver

proteins of rats given N^{15}-2-fluorenylacetamide and dialysis of aliquots of the homogenates of liver samples before separation of the soluble and insoluble fractions had no effect on the isotope content. There was evidence of some loss of isotopic nitrogen after treatment with urea, with Tween 80, with mercaptoethanol, and following reprecipitation by acid from hot NaOH solution. The differences, however, in the amounts of enrichment after the latter extractions were very slight.

In the preliminary study of the solubility of the isotopic nitrogen-containing material after alcoholic alkaline hydrolysis of pooled crude proteins, excess N^{15} was detectable only in the aqueous layer following repeated extractions of an aqueous suspension of the evaporated, unneutralized digest with ether. Attempts to further fractionate neutralized, *in vacuo*-dried alcoholic alkaline digests of fractions from the liver and from the small intestine resulted in the distribution of questionable enrichment in the aqueous and ether extracts and in the extracted residues.

In vitro *studies.*—With concentrations of N^{15}-2-fluorenamine of 50 μg. or of N^{15}-2-fluorenylacetamide of 35 μg. per ml. of substrate the protein fractions were found to contain 0.008 to 0.013 atom percent excesses of N^{15} over that of tank nitrogen, values that are similar to those of corresponding fractions isolated from the livers of rats following administration of the carcinogen. However, homogenates that had been heated in a boiling water bath for 5 minutes before transfer to the fluorenyl substrate sometimes yielded insoluble residues that were enriched to the same extent as fractions from unboiled homogenates, and the isotopic nitrogen could not be removed by additional re-extractions with organic solvents.

Relatively large concentrations of isotopic nitrogen were found in alcohol washes of the fractions, even from those that were isolated following prolonged water dialysis of the homogenates after incubation in the substrates.

The heat-coagulated protein from a 1.5 percent solution of crystalline bovine-serum albumin that had been incubated under the same experimental conditions and at the same time as rat-liver homogenates in substrates of N^{15}-2-fluorenamine or N^{15}-2-fluorenylacetamide bound insignificant amounts of isotopic nitrogen, if any, having values of 0.00463 atom percent excess N^{15} over tank nitrogen in acetate buffer alone and 0.00537 after incubation with the carcinogen. When the albumin was coagulated before suspension in the substrate and incubation no evidence of binding was detected.

Unlike the *in vivo* results with N^{15}-4-fluorenylacetamide, protein samples showed approximately the same enrichment with isotopic nitrogen after incubation with this relatively noncarcinogenic compound as after incubation with N^{15}-2-fluorenylacetamide. The acetate substrates contained 280 to 350 μg. of the 4-fluorenylacetamide and 50 to 70 μg. of the 2-derivative.

The protein fractions of homogenates and of slices from hepatoma 3683, transplant generation 43, and cholangioma 3924A,[10] after incubation in the

[10] We wish to thank Dr. H. Kahler and Mrs. Haines of this Institute for the A × C (Irish) strain rats carrying 38th-generation transplants of 3924A and for the Sprague-Dawley strain rats carrying 31st-generation intramuscular transplants of the Novikoff hepatoma.

labeled 2-fluorenamine or *N*-2-fluorenylacetamide, showed, approximately the same amount of enrichment with isotopic nitrogen as the corresponding fractions from the livers of the tumor-carrying A × C/9935(Irish) strain rats. In one series with materials from the Novikoff hepatoma,[11] little, if any, excess isotopic nitrogen was present in the isolated protein materials, and but little more in the coagulated soluble fractions from the livers of the Sprague-Dawley strain tumor-carrying rats, while the insoluble fractions from the livers were enriched to the maximum extent observed in these materials. This was true with both N^{15}-2-fluorenamine and N^{15}-2-fluorenylacetamide.

In 3 experiments in which slices or homogenates of guinea-pig liver and of rat liver were incubated with N^{15}-2-fluorenamine or N^{15}-2-fluorenylacetamide, control values for theg uinea-pig proteins from buffer alone varied from 0.00382 to 0.0055 and for the rat from 0.0060 to 0.0073. The proteins isolated from the N^{15}-labeled substrates from both guinea-pig and rat liver had ratios that varied from slightly above the control values to 0.003 atom percent excess isotopic nitrogen over that of their control fractions from buffer solution.

Discussion

Experimental evidence (*6,7*) has been cited to support the assumption that the nitrogen of *N*-2-fluorenylacetamide remains in the molecule during metabolism of the compound by the rat, at least with respect to the 9-carbon, so that proof of binding of the nitrogen by tissue proteins may be interpreted as evidence for the binding of the carcinogen or a metabolite. The first series of analyses, made 4 years ago, indicated that there was binding of isotopic nitrogen by the crude proteins isolated from the livers of rats following gastric administration of N^{15}-2-fluorenylacetamide. Experiments were started, using continuous feeding of the carcinogen for different periods of time or combining incorporation in the diet with administration by stomach tube, with the hope of effecting an increase in the amount of material bound. The results were disappointing but the details have been presented to support the conclusion that the nitrogen of *N*-2-fluorenylacetamide is bound to liver proteins in the rat and to demonstrate the serious limitations of the method due to the small amount of binding which takes place. The amounts of carcinogen accounted for by "protein bound" N^{15} in the liver are approximately as great as the amounts of azo dye reported to be bound by rat-liver protein in azo-dye carcinogenesis, but the large amount of protein nitrogen causes such dilution of the N^{15} of the carcinogen that the atom percent excess of isotopic nitrogen at maximum observed binding of the carcinogen is only 0.0045 greater than the concentration in control proteins from the livers of untreated rats. Such low $N^{15}:N^{14}$ ratios require tedious care in the analyses to avoid 1) contamination with the nitrogen from samples of higher N^{15} content and 2) to keep the air content of the samples extremely low.

During the course of this investigation one report has appeared in the literature in which the problem of protein binding has been studied with

[11] *See* footnote 10, p. 689.

material tagged with isotopic nitrogen. White, Cool, Stander, and David (*18*) observed a small accumulation of N^{15} in the livers of rats following the oral administration of *p*-dimethylphenylenediamine labeled with N^{15} in the free amine or the dimethylamine positions. The isotopic nitrogen disappeared from the liver in 24 and 48 hours. Since a large portion of the N^{15} could be removed by treatment of the alcohol-homogenized, defatted liver powder with hot trichloroacetic acid, the authors concluded that *most* of the compound found in the liver was definitely not bound to the liver protein in a stable protein-bound dye as is characteristic for *N, N*-dimethyl-*p*-phenylazo+aniline (*p*-dimethylaminoazobenzene).

The abundance of isotopic nitrogen in crude protein fractions isolated from the tissues of normal rats and guinea pigs was higher than that of standard tank nitrogen. The acetate and alcohol washes from the proteins of the tissues of normal rats also contained between 0.006 and 0.007 atom percent excess air-free N^{15} over that of standard tank nitrogen. The concentration of isotopic nitrogen was lower in the liver proteins of the untreated guinea pig than in those from the untreated rat. Such variations found in concentrations of isotopic nitrogen in biologic material are in agreement with observations reported by other investigators (*19, 20*).

Both protein fractions isolated from the livers of rats 6 and 24 hours after administration of N^{15}-2-fluorenylacetamide contained small but consistent excesses of isotopic nitrogen compared with the corresponding fractions from untreated rats. Enrichment was still detectable 1 week after administration of the carcinogen but questionable after 2 weeks. This decrease in so-called bound nitrogen during a period paralleling the "expected life" of liver proteins supports the conclusion that there is a union of the proteins and the nitrogen of the carcinogen.

Prolonged feeding of low but carcinogenic concentrations in the food resulted in somewhat lower ratios of isotopic to total nitrogen than did larger tolerated doses administered several times by stomach tube. Since the total amounts of carcinogen ingested were greater when incorporated in the food the results suggest that the rate of intake may have some influence on the amount of binding.

Twenty-four hours after administration of the last of 6 or of 13 repeated doses of N^{15}-2-fluorenylacetamide, 10 mg. per 100 gm. body weight during periods of 2 and 5 weeks, respectively, the amount of N^{15} calculated as fluorenylacetamide accounted for 0.07 to 0.15 μg. per mg. of protein fraction. Miller and Miller (*10*), finding protein-bound C^{14} distributed among the differentially centrifuged liver fractions from a rat fed *N*-2-fluoren-9-C^{14}-ylacetamide for 27 days, reported that total activity of the whole liver accounted for 77 μg. of the carcinogen. Miller and Miller (*21*) also recovered bound radioactivity from crude proteins of rat liver equal to 0.02 μg. fluorenylacetamide per mg. of protein. This amount of bound material is less than the minimum amount detectable by the isotopic-nitrogen procedure. The single dose administered by them, however, was approximately one fourth that administered repeatedly in this study, 22.7 mg. per kg. of body weight compared to 100 mg. The solvent and

the method of preparation of the protein fractions were also different. A quantitative comparison is, therefore, unjustified. Under experimental conditions similar to those used in the investigation of nitrogen binding, Weisburger, Weisburger, and Morris recovered bound radioactivity from the liver proteins equal to 0.06 to 0.09 μg. of *N*-2-fluorenylacetamide per mg. of protein 30 hours after a single dose (*22*) and a maximum of 0.19 μg. 24 hours after the last of 4 doses of 10, 10, 10, and 7.1 mg. of *N*-2-fluoren-9-C^{14}-ylacetamide to a rat (*9*).

Observations made with isotopic nitrogen are supported by the data of Weisburger, Weisburger, and Morris (*9,22*) with radioactive *N*-2-fluorenylacetamide: specifically, the calculated concentrations of bound fluorenylacetamide, the slightly greater concentrations in soluble than in insoluble protein fractions, and the decrease in the amount of bound material in liver proteins 1 week after the final dose of the carcinogen compared with the amount present after 24 hours. By partition of the activity from a hydrolysate of bound protein between ethereal and aqueous layers, they also found that the material carrying the activity was to a large extent of the water-soluble type (*9*), an observation which is in agreement with the evidence reported herein of the preferential water solubility of isotopic nitrogen after digestion of protein fractions with alcoholic KOH.

The low ratios of isotopic to total nitrogen found in liver fractions of rats following administration of N^{15}-2-fluorenylacetamide made it seem inadvisable to attempt to overcome the technical difficulties involved in the isolation of proteins of the normal mammary gland and the ductus acousticus externus, although the high incidence of *N*-2-fluorenylacetamide-induced tumors in these tissues would have made them interesting for study. Failure to detect concentrations of isotopic nitrogen significantly greater than those in the tissue of normal control rats in the protein fractions from lungs, heart, spleen, skin, and muscle following N^{15}-2-fluorenylacetamide may be due to the lack of sensitivity of the isotopic-nitrogen procedure. Weisburger, Weisburger, and Morris (*22*) recovered radioactivity in similarly prepared fractions from lungs, heart, spleen, kidneys, stomach, and small intestine following administration of a single dose of 9.18 mg. *N*-2-fluoren-9-C^{14}-ylacetamide. The radioactivity accounted for between 0.005 and 0.04 μg. of fluorenylacetamide per mg. of protein, concentrations that would give ratios of $N^{15} : N^{14}$ close to those of control fractions. The low ratios of $N^{15} : N^{14}$ in proteins from brain, primary mammary tumors, and transplanted liver tumors are unsuitable also for the detection of protein binding by the isotopic-nitrogen method. Since the liver proteins of the same rats showed some enrichment as a result of administration of the carcinogen it may be concluded that binding in these other tissues, if it does occur, is less than in the corresponding protein fractions from liver. The primary hepatoma appeared to have about the same small amount of enrichment as the fractions from surrounding liver. Before any significance can be attached to the low values for binding in the crude protein fractions

from primary and transplanted tumors, and indeed from many other tissues compared with liver proteins, more information must be available about the protein component(s) involved in the binding. Meanwhile, the possibility exists that a greater amount of binding occurs when a tissue is flooded with a carcinogen, a condition that may be limited to the liver following oral administration as a result of the absorbed material going directly from the intestinal tract to the liver by way of the portal vein.

The preliminary observation that the same maximum amount of binding of isotopic nitrogen of N^{15}-2-fluorenylacetamide by liver proteins occurred *in vitro* as *in vivo* suggested that the same biologic mechanism was involved. Subsequent experiments, however, showed occasional inconsistencies under supposedly identical experimental conditions. A possible explanation of these inconsistencies *in vitro* may be related to the extreme variation in the relative amounts of coagulable soluble protein found in the substrates. The time for bleeding the animals and for perfusion of the livers varied. The time of incubation and the temperature were kept constant and every effort was made to prevent autolysis by keeping the tissues in iced containers and by rapid homogenization or slicing. Greater extraction of soluble proteins would be expected to occur, however, with incubation for 3.5 hours than with the *in vivo* procedure of extraction for 1 to 1.5 hours in an ice bath. If any autolysis occurred it might increase the amount of coagulable soluble protein extracted and any protein-bound carcinogen present might be diluted enough to prevent detection of the N^{15}.

Unlike the results of the *in vivo* studies, enrichment of the isolated protein fractions was the same after incubation of rat-liver homogenates or slices with *N*-4-fluorenylacetamide as with the *N*-2-derivative and there was also enrichment of fractions from guinea-pig liver following incubation in the substrate. Heat-coagulated homogenates of rat liver sometimes showed enrichment after incubation with the isotopic nitrogen-tagged material. Under the same experimental conditions, bovine-serum albumin, before or after heat coagulation, did not show significant enrichment. Hydroxylation of *N*-2-fluorenylacetamide or 2-fluorenamine was not detected colorimetrically (*5*) in the substrates nor in the alcohol washes of the precipitated protein fractions following incubation of rat-liver homogenates or slices for 0.5, 3, or 6 hours. Weisburger (*23*) and Gutmann and co-workers (*24,25*) reported the binding of radioactivity from *N*-2-fluoren-9-C^{14}-ylacetamide by liver proteins *in vitro*. Gutmann, using incubation periods of 2 and 4 hours, concluded: 1) that oxidation of the carcinogen in the substrate was necessary to binding; 2) that slices showed greater ability to effect the oxidation than homogenates of liver; 3) that deacetylation, which occurs equally well in homogenates, was not necessary to binding. Weisburger demonstrated a progressive increase in the percentage of radioactivity incorporated in the proteins after incubation of liver homogenates in the substrate for 0, 2, and 22 hours under conditions where hydroxylation did not occur. A need for further investigation of protein binding of 2-fluorenylacetamide *in vitro* is indicated.

The absence of detectable enrichment with isotopic nitrogen of corresponding fractions from guinea-pig liver, following administration of N^{15}-2-fluorenylacetamide is in agreement with the observation of Miller and Miller (*21*) that the livers of guinea pigs had approximately one tenth as much protein-bound C^{14} as rat liver following administration of *N*-2-fluoren-9-C^{14}-ylacetamide. This finding is interesting since the guinea pig has been found to be relatively resistant to the carcinogenic action of *N*-2-fluorenylacetamide (*12,21,26*) or 2-fluorenamine (*27*).

The finding of similar enrichment of corresponding liver fractions from rats after administration of N^{15}-2-fluorenyldiacetamide and N^{15}-2-nitrofluorene as with N^{15}-2-fluorenylacetamide is not surprising since there is some evidence that these carcinogenic fluorene derivatives have a common metabolic pathway (*28*).

The failure of N^{15}-4-fluorenylacetamide to cause enrichment of the liver proteins with isotopic nitrogen following administration to the rat indicates that this compound or a metabolite is not bound under the same experimental conditions as the 2-derivative. Since 4-fluorenylacetamide has been found to show little, if any, carcinogenicity when compared with 2-fluorenylacetamide (*12*) the failure to show binding appears to support the view that protein binding is associated with carcinogenesis. With few exceptions excellent correlation has been found between the protein binding of azo dyes and the carcinogenicity of the azo-dye hepatocarcinogens (*21*) and between the protein binding of radioactive carbon in the skin of mice and the carcinogenic activity of a number of hydrocarbons (*29,30*). Heidelberger and Moldenhauer (*30*) found also that inhibitors of carcinogenic activity of several hydrocarbons inhibited protein binding of the carcinogens. Exceptions have been observed, however, where structurally related noncarcinogenic compounds have been bound to the protein material (*21,30*). With noncarcinogenic dibenz[*a,c*]anthracene suggestive evidence was obtained that the sites of protein binding were different from those for the carcinogen dibenz[*a,h*]anthracene (*30*). Thus, with the facts available at this time it would be unreasonable to conclude that evidence for the binding of noncarcinogenic compounds with crude protein fractions precludes the theory of protein binding as a prerequisite to carcinogenesis. Proof, on the other hand, that a carcinogenic agent was not bound by the target-tissue protein would invalidate the theory but such evidence has not been reported.

Since enrichment occurs in both the acetate-soluble and the insoluble fractions of the liver proteins, the binding cannot be limited to a specific protein. A recent report of Heidelberger and Davenport (*31*) that 6 protein fractions from the skin of the mouse show binding of radioactivity after application of dibenz[*a,h*]anthracene-9,10-C^{14} serves to emphasize the possibility, recognized by Miller and Miller (*21*), that not all the proteins that show binding need necessarily play a role in the carcinogenic reaction. In this connection, it is interesting that Sorof, Young, and Vogt (*32*) have found that one fraction of the electrophoretically resolved dye-binding "h" class of the soluble proteins of rat liver shows not only binding

of the dye but also an *increase* in the relative amount of the subcomponent with the feeding of 4-dimethylaminoazobenzene or its 3′-methyl derivative. The carcinogens had been fed for 32 and 18 to 21 days, respectively. If "protein deletion" (*21*) should prove to be essential in the carcinogenic process a *decrease* in the amount of this subcomponent would be expected to occur in a later stage of carcinogenesis.

With the newer knowledge that the accumulated data from several sources have provided on the problem of the protein binding of carcinogens, it seems that the most important need at present to the understanding of the phenomenon with regard to the aminofluorene compounds is the discovery, identification, and characterization of the behavior of a specific protein involved in the carcinogenic activity. Unless such a specific protein should be found to bind greater amounts of the labeled carcinogen than the crude protein fractions that have been analyzed, further studies of protein binding of *N*-2-fluorenylacetamide by the isotopic nitrogen method would seem to be inadvisable.

Summary

The possibility of the binding of *N*-2-fluorenylacetamide, or a metabolite, by proteins of the tissues of rats was investigated by determining the N^{15}:N^{14} ratios of isolated crude protein fractions following oral or gastric administration of the isotopic nitrogen-tagged compound.

Small but consistent excesses in the concentration of isotopic nitrogen were found in acetate-soluble and acetate-insoluble protein fractions from rat liver, but not from guinea-pig liver following administration of N^{15}-2-fluorenylacetamide. Significant enrichment of the rat-liver proteins was found following N^{15}-2-fluorenyldiacetamide and 2-nitro-N^{15}-fluorene but not after the relatively noncarcinogenic N^{15}-4-fluorenylacetamide.

The ratios of isotopic to total nitrogen varied in acetate-soluble and acetate-insoluble protein fractions from blood, brain, lungs, spleen, heart, kidneys, small intestine, skin, and muscle from different groups of rats following administration of N^{15}-2-fluorenylacetamide. The amount of enrichment, if there was any, was with rare exceptions less than that of the fractions from the liver of the host rat. The enrichment of liver protein fractions with N^{15} following administration of N^{15}-2-fluorenylacetamide to rats bearing the tumors was also greater than that of the proteins from primary mammary cancer, and from transplanted hepatoma and cholangioma, although there was evidence of some enrichment in the transplanted liver tumors.

A similar small concentration of isotopic nitrogen was found in corresponding protein fractions isolated from homogenates and slices of liver that had been incubated in acetate buffer solution saturated, when hot, with N^{15}-2-fluorenylacetamide, N^{15}-2-fluorenamine, or 2-nitro-N^{15}-fluorene. The combination of the isotopic nitrogen of these carcinogenic 2-fluorenyl derivatives with the liver proteins *in vitro* appears to be relatively nonspecific, showing similar enrichment with rat liver, guinea-

pig liver, preboiled homogenates of rat liver, and rat liver incubated with N^{15}-4-fluorenylacetamide.

The experimental data support the conclusion that the nitrogen in position 2 of the molecule of administered N^{15}-2-fluorenylacetamide is present in stable combination with proteins of the liver of the rat.

The limitations involving the use of isotopic nitrogen in studies of protein binding have been discussed.

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ANNOUNCEMENTS

American Cancer Society Research and Personnel Grants

Application forms for grants in support of research and of personnel for research of the American Cancer Society will be available early in November of 1956. Applications received by the Society by January 1, 1957, will be acted on during the late winter and early spring. Grants recommended will normally become effective July 1, 1957, unless some other arrangement is requested and approved.

Investigators now receiving support will hear directly from the Society concerning application for renewal grants.

Scientists interested in applying for new grants are invited to write to the American Cancer Society, Inc., 521 West 57th Street, New York 19, N. Y. Application blanks will be sent as soon as they are available.

In the future, application deadlines will be January 1, May 1, and September 1. Grants recommended will become effective 6 months after the application deadline.

American Cancer Society grants support research to increase knowledge about cancer. Applicants should state to what extent the studies on research they propose may accomplish this end.

Types of grants available will include Project Grants, Program Grants, Institutional Research Grants, Contracts for Research, Post-doctoral Fellowships, Grants for Scholars in Cancer Research, and Grants for Additional Faculty-level Positions.

National Institutes of Health—Applications for Research Grants

The Public Health Service makes grants to encourage and support research investigations and training in health—medical and dental, sanitary engineering, and allied fields. Applications for new research projects may be submitted to the Public Health Service at any time. Those received between November 1 and March 1 will be reviewed by the appropriate National Advisory Council in June. Those received between March 1 and July 1 will be reviewed in October or November. Those received between July 1 and November 1 will be reviewed in February or March. Notices of Council action will usually be mailed to applicants within 2 weeks after Council meetings.

Applications may be obtained by writing to the Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.

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The Journal of the National Cancer Institute is published monthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Twelve issues are published each year in two volumes, beginning with number 1 in July and January. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Journal of the National Cancer Institute, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

4. Insert each footnote in the text immediately after the line in which it is mentioned.

5. Type each table on a separate page, to be the page immediately following the paragraph in which the table is first mentioned.

6. Graphs, charts, and drawings are printed in the text and referred to as text-figures. Legends for text-figures should be included in the text immediately following the paragraph in which the text-figure is first mentioned. A drawing three times the size of that desired for the final figure is preferable. The lettering should be uniform and of such size that in the reproduction it will be approximately the same size as that of the printed type used in the text. The original drawings reproduce best and are therefore preferable. If two additional copies (photostats, etc.) of each drawing are also submitted, review of the manuscript is expedited.

7. Photographs are printed together at the end of each article, as plate pages. The maximum size for each printed plate page is 5¼ by 8 inches, and the photographs should be combined into as few plates as possible. Photographs should be submitted either in the exact size, or exactly double the size, of the final printed reproduction. Mount the photographs for each plate together on one piece of heavy cardboard. Number each photograph consecutively. Type the legends for each plate on a separate page. If, in addition, two unmounted duplicate prints of each photograph are also submitted, review of the manuscript will be expedited.

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 17 • NO. 5 • NOVEMBER 1956

Page

Dr. George Hall Hogeboom, 1913–1956 III

Histochemical characteristics of intrasplenic ovarian transplants in gonadectomized rats. *Helen Wendler Deane* and *Don W. Fawcett* 541

Observations on the morphology and histochemistry of the mouse pituitary implanted in the anterior eye chamber. *Eleanor R. Siperstein* and *Monte A. Greer* 569

Unusual neoplastic lesions of the uterine horns of estrogen-treated mice. *W. U. Gardner* and *M. Ferrigno* 601

The influence of succinate and 2,4-dinitrophenol on the respiratory activity of mouse cancers and normal tissues. *Mark Woods* 615

Combined treatment of advanced leukemia (L1210) in mice with amethopterin and 6-mercaptopurine. *John M. Venditti, Stewart R. Humphreys, Nathan Mantel,* and *Abraham Goldin* 631

Subcutaneous fibrosarcomas in strains C3H and C57BL female mice, and F_1 and backcross hybrids of these strains. *Thelma B. Dunn, Walter E. Heston,* and *Margaret K. Deringer* 639

Effect of *in vitro* X irradiation on pigmented and pale slices of Cloudman S91 mouse melanoma as measured by subsequent proliferation *in vivo*. *Jewell Plummer Cobb* 657

Recorded and expected mortality among the Chinese of Hawaii and the United States with special reference to cancer. *Robert L. Smith* 667

Studies on the protein binding of *N*-2-fluorenylacetamide. *Helen M. Dyer* and *Harold P. Morris* 677

Announcements:

American Cancer Society Research and Personnel Grants 699

National Institutes of Health—Applications for Research Grants 699

956

Page
III

541

569

601

615

31

39

57

67

77

99
99